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<b>(21) International Application Number:</b> PCT/EP96/03605 <b>(22) International Filing Date:</b> 14 August 1996 (14.08.96) <b>(30) Priority Data:</b> 95307332.7 16 October 1995 (16.10.95) GB <b>(71) Applicant (for all designated States except AU BB CA GB IE LK MN MW NZ SD US):</b> UNILEVER N.V. [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL). <b>(71) Applicant (for AU BB CA GB IE LK MN MW NZ SD only):</b> UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4 4BQ (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DAVIS, Paul, James [GB/GB]; The Hawthorns, Pavenham Road, Felmersham, Bedfordshire MK43 7EX (GB). VAN DER LOGT, Cornelis, Paul, Erik [NL/GB]; 1 Bluebell Rise (Peverel Manor Estate), Rushden, Northamptonshire MK43 7EX (GB). VERHOEIJEN, Martine, Elisa [BE/GB]; 1 Tintagel Close (Manor Farm Estate), Rushden, Northamptonshire NN10 0TU (GB). WILSON, Steve [GB/GB]; 3 Aldenham Close (Goldington), Bedford MK41 0FQ (GB).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> With international search report.	
<b>(54) Title:</b> A BIFUNCTIONAL OR BIVALENT ANTIBODY FRAGMENT ANALOGUE  <b>(57) Abstract</b>  The invention relates to a bispecific or bivalent antibody fragment analogue comprising a binding complex containing two polypeptide chains, whereby one polypeptide chain comprises two heavy chain variable domains (V <sub>H</sub> ) in series and the other polypeptide chain comprises two light chain variable domains (V <sub>L</sub> ) in series, the binding complex further containing two pairs of variable domains (V <sub>H</sub> -A//V <sub>L</sub> -A and V <sub>H</sub> -B//V <sub>L</sub> -B). The two V <sub>H</sub> 's and/or the two V <sub>L</sub> 's are connected directly or via an intermediate peptide linker. Also a production method for such antibody fragment analogues is disclosed.		

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Title: A bifunctional or bivalent antibody fragment analogue

The invention relates to new bispecific or bivalent antibody fragment analogues, a process for preparing such antibody fragment analogues and various uses of such antibody fragment analogues.

Background of the invention and prior art

10 1. Antibody structure

Antibody molecules typically are Y-shaped molecules whose basic unit consist of four polypeptides, two identical heavy chains and two identical light chains, which are covalently linked together by disulfide bonds. Each of these chains is folded in discrete domains. The C-terminal regions of both heavy and light chains are conserved in sequence and are called the constant regions, also known as C-domains. The N-terminal regions, also known as V-domains, are variable in sequence and are responsible for the antibody specificity. The antibody specifically recognizes and binds to an antigen mainly through six short complementarity-determining regions located in their V-domains (see Figure 1).

25 In this specification abbreviations are used having the following meaning.

C-domain:	Constant domain
V-domain:	Variable domain
V <sub>L</sub>	: Variable domain of the light chain
30 V <sub>H</sub>	: Variable domain of the heavy chain
Fv	: dual chain antibody fragment containing both a V <sub>H</sub> and a V <sub>L</sub>
scFv	: single-chain Fv (V <sub>H</sub> and V <sub>L</sub> genetically linked either directly or via a peptide linker)
35 CDR	: Complementarity-determining region
ELISA	: Enzyme Linked Immuno Sorbent Assay
PCR	: Polymerase Chain Reaction

IPTG : IsoPropyl- $\beta$ -ThioGalactopyranoside  
PBS : Phosphate Buffered Saline  
PBST : Phosphate Buffered Saline with 0.15% Tween  
TMB : 3,3',5,5'-TetraMethylBenzidine

5

It is generally known that proteolytic digestion of an antibody with papain yields three fragments. The fragment containing the CH<sub>2</sub> and CH<sub>3</sub> domains of the two heavy chains connected by the complete hinge (see Figure 1) crystallises very easily and was therefore called Fc fragment. The two other fragments are identical and were called Fab fragments, as they contained the antigen-binding site. Digestion with pepsin is such that the two Fab's remain connected via the hinge, forming only two fragments: Fc' and Fab<sub>2</sub>.

The Fv is the smallest unit of an antibody which still contains the complete binding site (see Figure 1) and full antigen binding activity. It consists of only the V-domains of the heavy and light chains thus forming a small, heterodimeric variable fragment or Fv. Fv's have a molecular weight of about 25 kD, which is only one sixth of the parent whole antibody (in the case of an IgG). Previously Fv's were only available by proteolysis in a select number of cases (Givol, 1991). The production of Fv's can now be achieved more routinely using genetic engineering methods through cloning and expressing DNA encoding only the V-domains of the antibody of interest. Smaller fragments, such as individual V-domains (Domain Antibodies or dABs, Ward et al., 1989), and even individual CDR's (Williams et al., 1989; Taub et al., 1989) were shown to retain the binding characteristics of the parent antibody. However, this is not achievable on a routine basis: most naturally occurring antibodies need both a V<sub>H</sub> and a V<sub>L</sub> to retain full immunoreactivity. For example, in the case of V<sub>H</sub> D1.3 (Ward et al., 1989), although it still binds hen egg lysozyme (HEL) with an affinity close to that of the parent antibody, it was shown that loss of

specificity was observed in that it can no longer distinguish turkey lysozyme from HEL, whereas the Fv can (Berry and Davies, 1992). Although murine dABs can be obtained more routinely from spleen libraries (Ward et al., 1989), the approach is unsustainable because of the many problems associated with their production and physical behaviour: expression is extremely poor, affinity tends to be low, stability and solubility in water is low, and non-specific binding is usually very high. According to the literature a possible explanation of these undesirable characteristics is the exposure of the hydrophobic residues which are normally buried in the  $V_H$ - $V_L$  interface. The exposed hydrophobic patches are thought to contribute to aggregation of the protein inside the cells and/or in the culture medium, leading to poor expression and/or poor solubility (Anthony et al., 1992; Ward et al., 1989). The hydrophobic patches can also explain the high non-specific binding described by Berry and Davies, 1992. These problems clearly limit the usefulness of these molecules.

Most of the Camelid antibodies appear to be an exception to this rule in that they only need one V-domain, namely  $V_H$ , to specifically and effectively bind an antigen (Hamers-Castermans et al., 1993). In addition, preliminary data indicate that they seem not to suffer from the disadvantages of mouse dABs, as these camelid antibodies or fragments thereof are soluble and have been shown to express well in yeast and *Aspergillus* moulds. These observations can have important consequences for the production and exploitation of antibody-based products, see patent application WO 94/25591 (UNILEVER et al., first priority date 29.04.93).

## 2. Production of antibody fragments

Several microbial expression systems have already been developed for producing active antibody fragments, e.g. the production of Fab in various hosts, such as *E. coli* (Better et al., 1988, Skerra and Plückthun, 1988, Carter et al.,

1992), yeast (Horwitz et al., 1988), and the filamentous fungus *Trichoderma reesei* (Nyyssönen et al., 1993) has been described. The recombinant protein yields in these alternative systems can be relatively high (1-2 g/l for Fab  
5 secreted to the periplasmic space of *E. coli* in high cell density fermentation, see Carter et al., 1992), or at a lower level, e.g. about 0.1 mg/l for Fab in yeast in fermenters (Horwitz et al., 1988), and 150 mg/l for a fusion protein CBHI-Fab and 1 mg/l for Fab in *Trichoderma*  
10 in fermenters (Nyyssönen et al., 1993) and such production is very cheap compared to whole antibody production in mammalian cells (hybridoma, myeloma, CHO). Although the latter can give yields of the order of 1 g/l in high cell density fermentation, it is a time-consuming and very  
15 expensive manufacturing method resulting in a cost price of about 1000 £/gram of antibody. It was further demonstrated that plants can be used as hosts for the production of both whole antibodies (Hiatt et al., 1989) and scFv's (Owen et  
al., 1992, Firek et al., 1993), whereby yields of upto 0.5%  
20 of the total soluble protein content in tobacco leaves were mentioned.

The fragments can be produced as Fab's or as Fv's, but additionally it has been shown that a  $V_H$  and a  $V_L$  can be genetically linked in either order by a flexible  
25 polypeptide linker, which combination is known as an scFv (Bird et al. (1988), Huston et al. (1988), and granted patent EP-B-0281604 (GENEX/ENZON LABS INC.; first priority date 02-09-1986).

### 30 3. Bivalent and bispecific antibodies and antibody fragments

The antibody fragments Fab, Fv and scFv differ from whole antibodies in that the antibody fragments carry only a single antigen-binding site. Recombinant fragments with two  
35 binding sites have been made in several ways, for example, by chemical cross-linking of cysteine residues introduced at the C-terminus of the  $V_H$  of an Fv (Cumber et al., 1992),

or at the C-terminus of the  $V_L$  of an scFv (Pack and Plückthun, 1992), or through the hinge cysteine residues of Fab's (Carter et al., 1992). Another approach to produce bivalent antibody fragments is described by Kostelny et al.  
5 (1992) and Pack and Plückthun (1992) and is based on the inclusion of a C-terminal peptide that promotes dimerization.

When two different specificities are desired, one can generate bispecific antibody fragments. The traditional  
10 approach to generate bispecific whole antibodies was to fuse two hybridoma cell lines each producing an antibody having the desired specificity. Because of the random association of immunoglobulin heavy and light chains, these hybrid hybridomas produce a mixture of up to 10 different  
15 heavy and light chain combinations, only one of which is the bispecific antibody (Milstein and Cuello, 1983). Therefore, these bispecific antibodies have to be purified with cumbersome procedures, which considerably decrease the yield of the desired product.

20 Alternative approaches include in-vitro linking of two antigen specificities by chemical cross-linking of cysteine residues either in the hinge or via a genetically introduced C-terminal Cys as described above. An improvement of such in vitro assembly was achieved by using  
25 recombinant fusions of Fab's with peptides that promote formation of heterodimers (Kostelny et al., 1992). However, the yield of bispecific product in these methods is far less than 100%.

A more efficient approach to produce bivalent or bispecific  
30 antibody fragments, not involving in vitro chemical assembly steps, was described by Holliger et al. (1993). This approach takes advantage of the observation that scFv's secreted from bacteria are often present as both monomers and dimers. This observation suggested that the  $V_H$   
35 and  $V_L$  of different chains can pair, thus forming dimers and larger complexes. The dimeric antibody fragments, also named "diabodies" by Hollinger et al., in fact are small

bivalent antibody fragments that assembled in vivo. By linking the  $V_H$  and  $V_L$  of two different antibodies 1 and 2, to form "cross-over" chains  $V_{H1}V_{L2}$  and  $V_{H2}V_{L1}$  (see Figure 2B), the dimerisation process was shown to reassemble both antigen-binding sites. The affinity of the two binding sites was shown to be equal to the starting scFv's, or even to be 10-fold increased when the polypeptide linker covalently linking  $V_H$  and  $V_L$  was removed, thus generating two proteins each consisting of a  $V_H$  directly and covalently linked to a  $V_L$  not pairing with the  $V_H$  (see Figure 2C). This strategy of producing bispecific antibody fragments was also described in several patent applications. Patent application WO 94/09131 (SCOTGEN LTD; priority date 15.10.92) relates to a bispecific binding protein in which the binding domains are derived from both a  $V_H$  and a  $V_L$  region either present at two chains or linked in an scFv, whereas other fused antibody domains, e.g. C-terminal constant domains, are used to stabilise the dimeric constructs. Patent application WO 94/13804 (CAMBRIDGE ANTIBODY TECHNOLOGY / MEDICAL RESEARCH COUNCIL; first priority date 04.12.92) relates to a polypeptide containing a  $V_H$  and a  $V_L$  which are incapable of associating with each other, whereby the V-domains can be connected with or without a linker.

Mallender and Voss, 1994 (also described in patent application WO 94/13806; DOW CHEMICAL CO; priority date 11.12.92) reported the in vivo production of a single-chain bispecific antibody fragment in *E. coli*. The bispecificity of the bivalent protein was based on two previously produced monovalent scFv molecules possessing distinct specificities, being linked together at the genetic level by a flexible polypeptide linker. The thus formed  $V_{H1}$ -linker- $V_{L1}$ -linker- $V_{H2}$ -linker- $V_{L2}$  fragment (see Figure 2A) was shown to contain both antigen binding specificities 1 and 2. (1= anti-fluorescein, 2= anti-single-stranded DNA).



Traditionally, whenever single-chain antibody fragments are referred to, a single molecule consisting of one heavy chain linked to one (corresponding) light chain in the presence or absence of a polypeptide linker is implicated.

- 5 When making bivalent or bispecific antibody fragments through the 'diabody' approach (Holliger et al., (1993) and patent application WO 94/09131) or by the 'double scFv' approach (Mallender and Voss, 1994 and patent application WO 94/13806), again the  $V_H$  is linked to a (the
- 10 corresponding)  $V_L$ .

- It is realised that claims 32 and 33 of patent application WO 93/11161 (ENZON INC.; priority date 25.11.91) and the corresponding passages in that specification on page 22, lines 1-10 may read on a polypeptide comprising two  $V_L$ 's
- 15 fused together via a flexible polypeptide linker, and on a polypeptide comprising two  $V_H$ 's fused together via a flexible polypeptide linker, respectively. However, no examples were given to substantiate this approach, thus it was in fact a hypothetical possibility instead of an
- 20 actually produced compound.

- A skilled person would not have expected that such approach would be viable for at least three reasons. Firstly, it is widely recognised that immunoglobulin heavy chains (excluding the above described camel immunoglobulins) have
- 25 very limited solubility and spontaneously precipitate out of aqueous solution when isolated from their light chain partners. Secondly, several groups have shown (Ward et al., 1989, Berry and Davies, 1992, and Anthony et al., 1992) that expression of  $V_H$ 's in the absence of  $V_L$ 's is hampered
- 30 by extremely poor yields of unstable product with many undesirable properties, e.g. non-specific binding. Thirdly in patent application WO 94/13804 it was described on page 31 lines 10-12, that in computer modelling experiments they could not model as heterodimers  $V_H$ - $V_H$  and  $V_L$ - $V_L$  given the
- 35 constraints of short linkers.

Thus the simple suggestion given in patent application WO 93/11161 is not an enabling disclosure leading a skilled

person to try with a reasonable expectation of success whether such suggestion would work; therefore, that patent application should not be considered as relevant prior art for the present invention.

5

### Summary of the invention

The present invention provides a **bispecific** or **bivalent** antibody fragment analogue, which comprises a binding complex containing two polypeptide chains, one of which  
10 comprises two times a variable domain of a heavy chain ( $V_H$ ) **in series** and the other comprises two times a variable domain of a light chain ( $V_L$ ) **in series**.

In one aspect of the invention one chain of the antibody fragment analogue comprises a first  $V_H$  ( $V_H$ -A) connected to a  
15 second  $V_H$  ( $V_H$ -B) and the other chain comprises a first  $V_L$  ( $V_L$ -A) connected to a second  $V_L$  ( $V_L$ -B). In a preferred embodiment of this aspect one chain comprises a first  $V_H$  ( $V_H$ -A) followed by a second  $V_H$  ( $V_H$ -B), thus [ $V_H$ -A \*  $V_H$ -B], and the other chain comprises a first  $V_L$  ( $V_L$ -A) preceded by  
20 a second  $V_L$  ( $V_L$ -B), thus [ $V_L$ -B \*  $V_L$ -A]. For some embodiments of this aspect the two  $V_H$ 's are directly connected to each other, but for other embodiments of this aspect of the invention the two  $V_L$ 's are directly connected to each other. According to another embodiment of this aspect of the  
25 invention the two  $V_H$ 's are connected to each other by a linker and also the two  $V_L$ 's are connected to each other by a linker. Such a linker usually comprises at least one amino acid residue.

According to a special embodiment of this aspect of the  
30 invention one chain comprises a first  $V_H$  ( $V_H$ -A) followed by a second  $V_H$  ( $V_H$ -B), thus [ $V_H$ -A \*  $V_H$ -B], and the other chain comprises a first  $V_L$  ( $V_L$ -A) followed by a second  $V_L$  ( $V_L$ -B), thus [ $V_L$ -A \*  $V_L$ -B], and in which the two  $V_H$ 's are connected to each other by a linker and also the two  $V_L$ 's are  
35 connected to each other by a linker, whereas each linker comprises at least 10 amino acid residues.

According to the above aspect of the invention with A being different from B there are provided **bispecific** antibody fragment analogues.

According to another aspect of the invention the  
5 specificities A and B are the same resulting in a **bivalent** antibody fragment.

According to a further aspect of the invention the bispecific or bivalent antibody fragment analogues can be used in a diagnostic technique or for immunoassays, in a  
10 purification method, for therapy, or in other methods in which immunoglobulins or fragments thereof are used. Such uses are well-known in the art.

The invention also provides a process for producing the antibody fragments of the invention in that a host is  
15 transformed by incorporating into that host a DNA encoding the two  $V_H$ 's with or without a connecting linker and a DNA encoding the two  $V_L$ 's with or without a connecting linker. Preferably the two DNA's are placed in a dicistronic arrangement.

20 It is also possible that the two linked  $V_H$ 's and the two linked  $V_L$ 's are produced separately by different hosts, after which the linked  $V_H$ 's produced by one host can be combined with the linked  $V_L$ 's produced by the other host. The hosts can be selected from the group consisting of  
25 prokaryotic bacteria of which examples are Gram-negative bacteria, e.g. *E. coli*, and Gram-positive bacteria, e.g. *B. subtilis* or lactic acid bacteria, lower eukaryotes examples of which are yeasts, e.g. belonging to the genera *Saccharomyces*, *Kluyveromyces*, or *Trichoderma*, moulds, e.g.  
30 belonging to the genera *Aspergillus* and *Neurospora*, and higher eukaryotes, examples of which are plants, e.g. tobacco, and animal cells, examples of which are myeloma cells and CHO cells. The techniques to transform a host by genetic engineering methods in order to have a desirable  
35 polypeptide produced by such host are well-known to persons skilled in the art as is evident from the literature

mentioned above under the heading "Background of the invention and prior art".

Brief description of the drawings

- 5 Figure 1 depicts in schematic form the structure of a typical antibody (immunoglobulin) molecule.
- Figure 2 shows a schematic representation of published arrangements of heavy and light chain V-domain gene fragments that have been proven to
- 10 produce bispecific antibody fragments.
- Figure 3 shows in diagrammatic form the suggested arrangement of the V-domains of a double head antibody fragment according to the invention with the V-domains in the following order:
- 15  $V_HA-V_{HB} + V_{LB}-V_{LA}$ .
- Figure 4 shows the nucleotide sequence of the *EcoRI*-*HindIII* insert of pUR.4124 containing DNA (see SEQ ID NO: 1) encoding  $V_L$ Lys-Linker- $V_H$ Lys (see SEQ ID NO: 2).
- 20 Figure 5 shows the nucleotide sequence of the *HindIII*-*EcoRI* insert of plasmid Fv.3418 (see SEQ ID NO: 3) containing DNA encoding pelB leader- $V_H$ 3418 (see SEQ ID NO: 4) and DNA encoding pelB leader- $V_L$ 3418 (see SEQ ID NO: 5).
- 25 Figure 6 shows the nucleotide sequence of the *HindIII*-*EcoRI* insert of plasmid Fv.4715-myc (see SEQ ID NO: 6) containing DNA encoding pelB leader- $V_H$ 4715 (see SEQ ID NO: 7) and DNA encoding pelB leader- $V_L$ 4715-Myc tag (see SEQ ID NO: 8).
- 30 Figure 7 shows the nucleotide sequence of the *HindIII*-*EcoRI* insert of scFv.4715-myc containing DNA (see SEQ ID NO: 9) encoding pelB leader- $V_H$ 4715-Linker- $V_L$ 4715-Myc tag (see SEQ ID NO: 10).
- 35 Figure 8 a/b shows the nucleotide sequence of the *HindIII*-*EcoRI* insert of pGOSA.E (see SEQ ID NO: 11) containing DNA encoding pelB leader- $V_H$ 4715-

Linker-V<sub>L</sub>3418 (see SEQ ID NO: 12) and DNA encoding pelB leader-V<sub>L</sub>3418-Linker-V<sub>H</sub>4715 (see SEQ ID NO: 13).

- Figure 9 gives an overview of the oligonucleotides and their positions in pGOSA.E that can be used to replace V-domain gene fragments.
- Figure 10 illustrates the amino acid sequence of the V<sub>H</sub>-V<sub>H</sub> and V<sub>L</sub>-V<sub>L</sub> domain junctions in fusion polypeptides GOSA.E (see amino acids 114-145 in SEQ ID NO: 12 and amino acids 102-128 in SEQ ID NO: 13), GOSA.V (see SEQ ID NO: 30 and amino acids 102-128 in SEQ ID NO: 13), GOSA.S (see amino acids 114-145 in SEQ ID NO: 12 and SEQ ID NO: 31) and GOSA.T (see SEQ ID NO: 30 and SEQ ID NO: 31).
- Figure 11 shows the specificity of *Streptococcus* binding of scFv.4715-myc.
- Figure 12 shows the specificity of glucose oxidase targeting onto the surface of various *Streptococcus* strains by GOSA.E.
- Figure 13 shows the specificity of glucose oxidase targeting onto the surface of various *Streptococcus* strains by GOSA.V.
- Figure 14 shows the specificity of glucose oxidase targeting onto the surface of various *Streptococcus* strains by GOSA.S.
- Figure 15 shows the specificity of glucose oxidase targeting onto the surface of various *Streptococcus* strains by GOSA.T.
- Figure 16 shows the results of an ELISA. Individual fractions of a gelfiltration experiment using partially purified GOSA.E as feedstock were tested for glucose oxidase and *Streptococcus sanguis* bispecific binding activity.
- Figure 17 shows the results of an ELISA. Individual fractions of a gelfiltration experiment using partially purified GOSA.V as feedstock were

tested for glucose oxidase and *Streptococcus sanguis* bispecific binding activity.

- Figure 18 shows the results of an ELISA. Individual fractions of a gelfiltration experiment using partially purified GOSA.S as feedstock were tested for glucose oxidase and *Streptococcus sanguis* bispecific binding activity.
- Figure 19 shows the results of an ELISA. Individual fractions of a gelfiltration experiment using partially purified GOSA.T as feedstock were tested for glucose oxidase and *Streptococcus sanguis* bispecific binding activity.
- Figure 20 shows the source of fragment PCR.I *BstEII/SacI*
- Figure 21 shows the source of fragment PCR.II *SfiI/EcoRI*
- Figure 22 shows the source of fragment PCR.III *NheI/SacI*
- Figure 23 shows the source of fragment PCR.IV *XhoI/EcoRI*
- Figure 24 shows the source of fragment PCR.V *SalI/EcoRI*
- Figure 25 shows the source of fragment PCR.VI *SfiI/NheI*
- Figure 26 shows the source of fragment PCR.VII *BstEII/NheI*
- Figure 27 shows the source of fragment PCR.VIII *XhoI/EcoRI*
- Figure 28 shows the source of fragment PCR.IX *BstEII/NheI*
- Figure 29 shows the source of fragment PCR.X *PstI/EcoRI*
- Figure 30 shows the construction of plasmid pGOSA.A
- Figure 31 shows the construction of plasmid pGOSA.B
- Figure 32 shows the construction of plasmid pGOSA.C
- Figure 33 shows the construction of plasmid pGOSA.D
- Figure 34 shows the construction of plasmid pGOSA.E
- Figure 35 shows the construction of plasmid pGOSA.V
- Figure 36 shows the construction of plasmid pGOSA.S
- Figure 37 shows the construction of plasmid pGOSA.T
- Figure 38 a/b shows the construction of plasmid pGOSA.G
- Figure 39 shows the construction of plasmid pGOSA.J
- Figure 40 shows the construction of plasmid pGOSA.Z
- Figure 41 shows the construction of plasmid pGOSA.AA

- Figure 42 shows the construction of plasmid pGOSA.AB  
Figure 43 shows the construction of plasmid pGOSA.L  
Figure 44 shows the construction of plasmid pGOSA.Y  
Figure 45 shows the construction of plasmid pGOSA.X  
5 Figure 46 shows the construction of plasmid pGOSA.AC  
Figure 47 shows the construction of plasmid pGOSA.AD.

Table 1 shows the nucleotide sequence of the  
oligonucleotides used to produce the constructs  
10 described in this specification. Restriction sites  
encoded by these primers are underlined.

Table 2 gives an overview of all GOSA constructs described  
in this specification.

15 Table 2A describes intermediate constructs that  
were not further tested.

Table 2B describes the dicistronic constructs.

Table 2C describes the monocistronic constructs.

20 **Detailed description of the invention**

In this specification the construction of an antibody  
fragment analogue consisting of a two chain protein complex  
is described, in which one of the chains consists of two  
heavy chain V-domains and the other chain consists of the  
25 two corresponding light chain V-domains in either order.  
The variable domains are linked either directly or through  
a polypeptide linker. Subsequent molecular modelling of  
this combination suggested that the protein chains could  
fold such that both binding sites are fully accessible,  
30 provided that the connecting linkers are kept long enough  
to span 30 to 35 Å.

Whereas in patent application WO 93/11161 it is explicitly  
described that for the above described bispecific complexes  
two flexible polypeptide linkers in the self assembling  
35 complex are required, the present invention illustrated  
here describes in particular the construction of a two  
chain protein complex containing only one linker or no

linkers at all. The latter antibody fragment analogue thus consists of a two chain protein complex containing one polypeptide chain comprising heavy chain V-domains fused directly together and another polypeptide chain comprising the corresponding light chain V-domains fused together, both fusions in the absence of linkers. But also two chain protein complexes in which each chain comprises a linker between the two variable domains can be used as antibody fragment analogues according to the invention as described below with construct pGOSA.E. However, the two chain complexes containing only one linker or no linker at all are preferred. The abbreviation GOSA used in this specification relates to a combination of glucose oxidase and *Streptococcus sanguis*.

In this specification evidence is provided that these antibody fragment analogues ("double heads") contain both antigen binding specificities of the Fv's used to generate these bispecific antibody fragments. It is exemplified that these type of constructs according to the invention can be used to target the enzyme glucose oxidase to whole bacteria, using antibody fragments derived from hybridomas expressing antibodies directed against these antigens.

The present invention is now described by reference to some specific examples, which are included for purposes of illustration only and are not intended to limit the scope of the invention.

### EXAMPLES

#### General experimental

##### **Strains, Plasmids and Media**

All cloning steps were performed in *E. coli* JM109 (*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*(*r<sub>k</sub>*, *m<sub>k</sub>*<sup>+</sup>), *relA1*, *supE44*,  $\Delta$ (*lac-proAB*), [*F'*, *traD36*, *proAB*, *lacI*<sup>q</sup> $\Delta$ M15]). *E. coli* cultures were grown in 2xTY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per litre H<sub>2</sub>O), where indicated



supplemented with 2% glucose and/or 100 µg/ml ampicillin. Transformations were plated out on SOBAG plates (20 g tryptone, 5 g yeast extract, 15 g agar, 0.5 g NaCl per litre H<sub>2</sub>O plus 10 mM MgCl<sub>2</sub>, 2% glucose, 100 µg/ml ampicillin) The expression vectors used are derivatives of pUC19. The oligonucleotide primers used in the PCR reactions were synthesized on an Applied Biosystems 381A DNA Synthesiser by the phosphoramidite method.

#### 10 Expression of GOSA constructs

Colonies from freshly transformed JM109 plated onto SOBAG plates were used to inoculate 2xTY medium supplemented with 100 µg/ml ampicillin, 2% glucose. Cultures were shaken at 37°C to an OD<sub>600</sub> in the range of 0.5 to 1.0. Cells were pelleted by centrifugation and the supernatant was removed. The pelleted cells were resuspended in 2xTY medium with 100 µg/ml ampicillin, 1 mM IPTG, and grown for a further 18 hours at 25°C. Cells were pelleted by centrifugation and the supernatant, containing the secreted chains, used directly in an ELISA. The proteins in the periplasm of the pelleted cells were extracted by resuspending the cell pellet in 1/20 of the original culture volume of lysis buffer (20% sucrose, 200 mM Tris-HCl pH 7.5, 1 mM EDTA, 500 µg/ml lysozyme). After incubation at 25°C for 20 minutes an equal volume of H<sub>2</sub>O was added and the incubation was continued for another 20 minutes. The suspension was spun at 10.000 g for 15 minutes and the supernatant containing the periplasmic proteins was used directly in an ELISA.

#### 30 ELISA

96 well ELISA plates (Greiner HC plates) were activated overnight at 37°C with 200 µl/well of an 1/10 dilution of an over night culture of *Streptococcus* cells in 0.05 M sodium carbonate buffer at pH=9.5. Following one wash with PBST, the antigen sensitised plates were pre-blocked for 1 hour at 37°C with 200 µl/well blocking buffer (2% BSA, 0.15% Tween in PBS). Samples containing 50 µl blocking

buffer plus 50  $\mu$ l culture supernatants or periplasmic cell extracts (neat or diluted with PBS) were added to the *Streptococcus* sensitised plate and incubated for 2 hours at 37°C. Following 4 washes with PBS-T, 100  $\mu$ l of blocking  
5 buffer containing glucose oxidase (50  $\mu$ g/ml) was added to every well. After incubation at 37°C for 1 hour unbound glucose oxidase was removed by 4 washes with PBS-T. Bound glucose oxidase was detected by adding 100  $\mu$ l substrate to  
10 each well (70 mM Na-citrate, 320 mM Na-phosphate, 27 mg/ml glucose, 0.5  $\mu$ g/ml HRP, 100  $\mu$ g/ml TMB). The colour reaction was stopped after 1 hour by the addition of 35  $\mu$ l 2 M HCl and the A450 was measured (compare Figures 11/15).

#### Affinity purification of GOSA antibody fragments

15 GOSA.E, GOSA.V, GOSA.S and GOSA.T were partially purified by affinity chromatography. 100 ml periplasmic extract of each of these constructs was loaded onto a Glucose-oxidase-Sepharose column (CNBr-Sepharose, Pharmacia) prepared according to the manufacturer's instructions. After  
20 extensive washes with PBS the bound GOSA antibody fragments were eluted in 0.1M glycine buffer at pH=2.8. The fractions were neutralised with Tris and analysed by polyacrylamide gel electrophoresis followed by silver staining and tested for the presence of double head activity.

25

#### **EXAMPLE 1. Construction of the pGOSA double head expression vectors**

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30 In this Example the construction of a two chain protein complex is described, in which one of the chains consists of two heavy chain V-domains and the other chain consists of the two corresponding light chain V-domains. The variable domains are linked either directly or through a  
35 polypeptide linker. The expression vectors used are derivatives of a pUC19 derived plasmid containing a HindIII-EcoRI fragment that in the case of plasmid

scFv.4715-myc contains a DNA fragment encoding one pelB signal sequence fused to the N-terminus of the  $V_H$  that is directly linked to the corresponding  $V_L$  of the antibody through a connecting flexible peptide linker, (Gly<sub>4</sub>Ser),  
5 (present in SEQ ID NO: 2 as amino acids 109-123 and in SEQ ID NO: 10 as amino acids 121-135), thus generating a single-chain molecule (see Figure 7).

In the dual-chain Fv and the pGOSA expression vectors, the DNA fragments encoding both the  $V_H$  and  $V_L$  of the antibody  
10 are preceded by a ribosome binding site and a DNA sequence encoding the pelB signal sequence in an artificial dicistronic operon under the control of a single inducible promoter (see Figures 5, 6 and 8). Expression of these constructs is driven by the inducible lacZ promoter. The  
15 nucleotide sequence of the *Hind*III-*Eco*RI inserts of the plasmids pUR.4124, Fv.3418, Fv.4715-myc and scFv.4715-myc constructs used for the generation of the bispecific antibody fragments are given in Figures 4-7, respectively. Moreover, a culture of *E. coli* cells harbouring plasmid  
20 scFv.4715-myc and a culture of *E. coli* cells harbouring plasmid Fv.3418 were deposited under the Budapest Treaty at the National Collection of Type Cultures (Central Public Health Laboratory) in London (United Kingdom) with deposition numbers NCTC 12916 and NCTC 12915, respectively.  
25 In agreement with Rule 28 (4) EPC, or a similar arrangement for a State not being a Contracting State of the EPC, it is hereby requested that a sample of such deposit, when requested, will be submitted to an expert only.

The construction of pGOSA.E (see Figure 8 for the *Hind*III-*Eco*RI insert of pUC19) involved several cloning steps. The  
30 appropriate restriction sites in the various domains were introduced by PCR directed mutagenesis using the oligonucleotides listed in Table 1 below. The pGOSA.E derivatives pGOSA.V, pGOSA.S and pGOSA.T with only one or  
35 no linker sequence are derived from the pGOSA.E construct by removing the linker sequences by means of PCR directed mutagenesis with oligonucleotides listed in Table 1 below.

Table 1.

DBL.1	5'-CAC CAT CTC CAG AGA CAA TGG CAA G-3'	(=SEQ ID NO: 14)
DBL.2	5'-GAG CGC GAG CTC <u>GGC CGA ACC GGC</u> C <sup>1</sup> GA TCC GCC	
5	ACC GCC AGA GCC-3'	(=SEQ ID NO: 15)
DBL.3	5'-CAG GAT CCG <u>GCC GGT TCG GCC</u> <sup>1</sup> CAG GTC CAG CTG	
	CAA CAG TCA GGA-3'	(=SEQ ID NO: 16)
DBL.4	5'-CTA CAT <u>GAA TTC</u> <sup>2</sup> <u>GCT AGC</u> <sup>3</sup> TTA TTA TGA GGA GAC	
	GGT GAC GGT GGT CCC TTG GC-3'	(=SEQ ID NO: 17)
10 DBL.5	5'-TAA TAA <u>GCT AGC</u> <sup>3</sup> GGA GCT GCA TGC AAA TTC TAT	
	TTC-3'	(= SEQ ID NO: 18)
DBL.6	5'-ACC AAG <u>CTC GAG</u> <sup>4</sup> ATC AAA CGG GG-3'	(= SEQ ID NO: 19)
DBL.7	5'-AAT GTC <u>GAA TTC</u> <sup>2</sup> <u>GTC GAC</u> <sup>5</sup> TCC GCC ACC GCC AGA	
	GCC-3'	(= SEQ ID NO: 20)
15 DBL.8	5'-ATT GGA <u>GTC GAC</u> <sup>5</sup> ATC GAA CTC ACT CAG TCT CCA	
	TTC TCC-3'	(= SEQ ID NO: 21)
DBL.9	5'-TGA AGT <u>GAA TTC</u> <sup>2</sup> <u>GCG GCC GC</u> <sup>6</sup> T TAT TAC CGT TTG	
	ATT TCG AGC TTG GTC CC-3'	(= SEQ ID NO: 22)
DBL.10	5'-CGA ATT <u>CGG TCA CC</u> <sup>8</sup> G TCT CCT CAC AGG TCC AGT	
20	TGC AAC AG-3'	(= SEQ ID NO: 23)
DBL.11	5'-CGA ATT <u>CTC GAG</u> <sup>4</sup> ATC AAA CGG GAC ATC GAA CTC	
	ACT CAG TCT CC-3'	(= SEQ ID NO: 24)
DBL.12	5'-CGA ATT <u>CGG TCA CC</u> <sup>8</sup> G TCT CCT CAC AGG TGC AGT	
	TGC AGG AG-3'	(= SEQ ID NO: 25)
25 PCR.51	5'-AGG T(C/G)(A/C) A(C/A) <u>C TGC AG</u> <sup>7</sup> (C/G) AGT C(A/T)G	
	G-3'	(= SEQ ID NO: 26)
PCR.89	5'-TGA GGA GAC <u>GGT GAC</u> C <sup>8</sup> GT GGT CCC TTG GCC CC-3'	
		(= SEQ ID NO: 27)
PCR.90	5'-GAC ATT <u>GAG CTC</u> <sup>9</sup> ACC CAG TCT CCA-3'	(= SEQ ID NO: 28)
30 PCR.116	5'-GTT AGA <u>TCT CGA G</u> <sup>4</sup> CT TGG TCC C-3'	(= SEQ ID NO: 29)
1 = <i>Sfi</i> I, 2 = <i>Eco</i> RI, 3 = <i>Nhe</i> I, 4 = <i>Xho</i> I, 5 = <i>Sal</i> I, 6 = <i>Not</i> I, 7 = <i>Pst</i> I, 8 = <i>Bst</i> EII, 9 = <i>Sac</i> I		

These three constructs lack some of the restriction sites at the new joining points. The  $V_HA-V_HB$  gene fragment without a linker lacks the 5'  $V_HB$  *SfiI* site. The  $V_LB-V_LA$  gene fragment without a linker lacks the 5'  $V_LA$  *SalI* site.

- 5 The position of the oligonucleotides in the pGOSA constructs given in Table 1 are shown in Figure 9. The pGOSA expression vectors and the oligonucleotides in Table 1 have been designed to enable most specificities to be cloned into the pGOSA constructs. Figure 10 shows the amino  
10 acid sequence of the junctions between the  $V_HA-V_HB$  and  $V_LB-V_LA$  fragments encoded by DNA present in pGOSA.E, pGOSA.V, pGOSA.S and pGOSA.T. A more detailed description of the preparation of pGOSA.E, pGOSA.V, pGOSA.S and pGOSA.T is given in Example 5.

15

**EXAMPLE 2. Bifunctional binding activity of GOSA double heads**

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- 20 In this Example we provide evidence that the above described molecules ("double heads"), i.e. the two chain protein complexes, contain both antigen binding specificities of the Fv's used to generate these multi-functional antibody fragment analogues. Figure 12-15 show  
25 that GOSA.E, GOSA.V, GOSA.S and GOSA.T can be used to specifically target the enzyme glucose oxidase to several *Streptococcus sanguis* strains using antibody fragments derived from hybridoma's expressing antibodies directed against these antigens.
- 30 Comparison of the binding specificity of the GOSA constructs (see Figures 12-15) and the binding specificity of the scFv.4715-myc (see Figure 11) shows that the fine specificity of the anti-*Streptococcus sanguis* scFv.4715 is preserved in the GOSA "double heads".

35

**EXAMPLE 3. FPLC analysis of GOSA double heads**

Partially purified GOSA.E, GOSA.V, GOSA.S and GOSA.T samples (estimated to be 50-80% pure by polyacrylamide gel electrophoresis) were analysed on a Pharmacia FPLC Superose 12 column. The analysis was performed using PBS at a flow rate of 0.3 ml/minute. Eluate was monitored at 280 nm and 0.3 ml fractions were collected and analysed by ELISA. Usually GOSA.E, GOSA.V, GOSA.S and GOSA.T samples only gave one GOSA double head activity peak as determined by ELISA (see Figures 16-19). The position of this peak in the elution pattern indicated that the molecular weight of the GOSA double head is 40-50 kD. Since this molecular weight corresponds to the expected molecular weight of the  $V_H2 + V_L2$  double head dimer, the conclusion is justified that GOSA.E, GOSA.V, GOSA.S and GOSA.T are primarily produced as dimeric molecules. Occasionally an activity peak with an apparent molecular weight of  $\approx 200$  kD was observed (see Figure 16). The presence of Glucose Oxidase activity in these fractions (data not shown) indicate that these fractions contain GOSA double head complexed with glucose oxidase that was eluted with the GOSA sample from the glucose oxidase-sepharose affinity matrix.

25

**EXAMPLE 4. Production of other double heads**

The methods described in the previous Examples were used to produce other double heads, which also appeared to be active against the antigens for which they were developed. These other double heads had the following specificities:

- anti-*S. sanguis* / anti-beta-HCG,
- anti-*S. sanguis* / anti-urease,
- anti-*S. sanguis* / anti-hen-egg-lysozyme,
- anti-beta-HCG / anti-hen-egg-lysozyme,
- anti-hen-egg-lysozyme / anti-glucose oxidase,
- anti-huIgG / anti-glucose oxidase,

anti-urease / anti-glucose oxidase,  
anti-lacto-peroxidase / anti-glucose oxidase,  
anti-alpha-HCG / anti-glucose oxidase, and  
anti-reactive-Red-6 / anti-glucose oxidase.

5

**EXAMPLE 5.** Detailed description of the preparation of  
intermediate constructs pGOSA.A, pGOSA.B  
pGOSA.C and pGOSA.D and their use for the  
preparation of plasmid pGOSA.E and its  
derivatives pGOSA.V, pGOSA.S and pGOSA.T

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10

#### Oligonucleotides and PCR

The primary structures of the oligonucleotide primers used  
in the construction of the bispecific 'pGOSA' constructs  
are shown in Table 1 above. Reaction mixture used for  
amplification of DNA fragments were 10 mM Tris-HCl, pH 8.3,  
2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin (w/v), 0.1% Triton X-  
100, 400 mM of each dNTP, 5.0 units of Vent DNA polymerase  
(New England Biolabs), 100 ng of template DNA, and 500 ng  
of each primer (for 100 µl reactions). Reaction conditions  
were: 94°C for 4 minutes, followed by 33 cycles of each 1  
minute at 94°C, 1 minute at 55°C, and 1 minute 72°C.

20

#### 25 Plasmid DNA\Vector\Insert preparation and ligation\transformation.

Plasmid DNA was prepared using the 'Qiagen P-100 Midi-DNA  
Preparation' system. Vectors and inserts were prepared by  
digestion of 10 µg (for vector preparation) or 20 µg (for  
insert preparation) with the specified restriction  
endonucleases under appropriate conditions (buffers and  
temperatures as specified by suppliers). Modification of  
the DNA ends with Klenow DNA polymerase and  
dephosphorylation with Calf Intestine Phosphorylase were  
performed according to the manufacturers instructions.  
Vector DNAs and inserts were separated through agarose gel  
electrophoresis and purified with DEAE-membranes NA45

30

35

(Schleicher & Schuell) as described by Maniatis et al. Ligations were performed in 20  $\mu$ l volumes containing 30 mM Tris-HCl pH 7.8, 10 mM  $MgCl_2$ , 10 mM DTT, 1 mM ATP, 300-400 ng vector DNA, 100-200 ng insert DNA and 1 Weiss unit  $T_4$  DNA ligase. After ligation for 2-4 h at room temperature,  $CaCl_2$  competent *E. coli* JM109 (Maniatis) were transformed using 7.5  $\mu$ l ligation reaction. The transformation mixtures were plated onto SOBAG plates and grown overnight at 37°C. Correct clones were identified by restriction analysis and verified by automated dideoxy sequencing (Applied Biosystems).

#### Restriction digestion of PCR products

Following amplification each reaction was checked for the presence of a band of the appropriate size by agarose gel electrophoresis. One or two 100  $\mu$ l PCR reaction mixtures of each of the PCR reactions PCR.I - PCR.X (Figure 20-29), together containing approximately 2-4  $\mu$ g DNA product were subjected to phenol-chloroform extraction, chloroform extraction and ethanol precipitation. The DNA pellets were washed twice with 70% ethanol and allowed to dry. Next, the PCR products were digested overnight (18 h) in the presence of excess restriction enzyme in the following mixes at the specified temperatures and volumes.

#### 25 PCR.I:

50 mM Tris-HCl pH 8.0, 10 mM  $MgCl_2$ , 50 mM NaCl, 4 mM spermidine, 0.4  $\mu$ g/ml BSA, 4  $\mu$ l (= 40 U) *SacI*, 4  $\mu$ l (= 40 U) *BstEII*, in 100  $\mu$ l total volume at 37°C.

#### PCR.II:

30 10 mM Tris-Acetate pH 7.5, 10 mM  $MgAc_2$ , 50 mM KAc (1x "One-Phor-All" buffer ex Pharmacia), 4  $\mu$ l (= 48 U) *SfiI*, in 50  $\mu$ l total volume at 50°C under mineral oil. After overnight digestion, PCR.II-*SfiI* was digested with *EcoRI* (overnight at 37°C) by the addition of 16  $\mu$ l  $H_2O$ , 30  $\mu$ l 10x  
35 "One-Phor-All" buffer (Pharmacia) (100 mM Tris-Acetate pH 7.5, 100 mM  $MgAc_2$ , 500 mM KAc) and 4  $\mu$ l (= 40 U) *EcoRI*.



PCR.III:

10 mM Tris-Acetate pH 7.5, 10 mM MgAc<sub>2</sub>, 50 mM KAc (1x "One-Phor-All" buffer {Pharmacia}), 4 µl (= 40 U) *NheI*, 4 µl (= 40 U) *SacI*, in 100 µl total volume at 37°C.

5 PCR.IV:

20 mM Tris-Acetate pH 7.5, 20 mM MgAc<sub>2</sub>, 100 mM KAc (2x "One-Phor-All" buffer {Pharmacia}), 4µl (= 40 U) *XhoI*, 4 µl (= 40 U) *EcoRI*, in 100 µl total volume at 37°C.

PCR.V:

10 20 mM Tris-Acetate pH 7.5, 20 mM MgAc<sub>2</sub>, 100 mM KAc (2x "One-Phor-All" buffer {Pharmacia}), 4 µl (= 40 U) *SalI*, 4 µl (= 40 U) *EcoRI*, in 100 µl total volume at 37°C.

PCR.VI:

10 mM Tris-Acetate pH 7.5, 10 mM MgAc<sub>2</sub>, 50 mM KAc (1x "One-Phor-All" buffer {Pharmacia}), 4 µl (= 48 U) *SfiI*, in 50 µl total volume at 50°C under mineral oil. After overnight digestion, PCR.VI-*SfiI* was digested with *NheI* (overnight at 37°C) by the addition of 41 µl H<sub>2</sub>O, 5 µl 10x "One-Phor-All" buffer (Pharmacia) (100 mM Tris-Acetate pH 20 7.5, 100 mM MgAc<sub>2</sub>, 500 mM KAc) and 4 µl (= 40 U) *NheI*.

PCR.VII:

50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 4 mM spermidine, 0.4 µg/ml BSA, 4 µl (= 40 U) *NheI*, 4 µl (= 40 U) *BstEII*, in 100 µl total volume at 37°C.

25 PCR.VIII:

20 mM Tris-Acetate pH 7.5, 20 mM MgAc<sub>2</sub>, 100 mM KAc (2x "One-Phor-All" buffer {Pharmacia}), 4 µl (= 40 U) *EcoRI*, in 50 µl total volume at 37°C. After overnight digestion, PCR.VIII-*EcoRI* was digested with *XhoI* (overnight at 37°) by 30 the addition of 46 µl H<sub>2</sub>O and 4 µl (= 40 U) *XhoI*.

PCR.IX:

25 mM Tris-Acetate, pH 7.8, 100 mM KAc, 10 mM MgAc, 1mM DTT (1x "Multi-Core" buffer {Promega}), 4 mM spermidine, 0.4 µg/ml BSA, 4 µl (= 40 U) *NheI*, 4 µl (= 40 U) *BstEII*, in 100 35 µl total volume at 37°C.

PCR.X:

50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 4 mM spermidine, 0.4 µg/ml BSA, 4 µl (= 40 U) *Pst*I, 4 µl (= 40 U) *Eco*RI, in 100 µl total volume at 37°C.

5

The digested PCR fragments

PCR.I-*Sac*I/*Bst*EII,

PCR.II-*Sfi*I/*Eco*RI,

PCR.III-*Nhe*I/*Sac*I,

PCR.IV-*Xho*I/*Eco*RI,

PCR.V-*Sal*I/*Eco*RI,

PCR.VI-*Sfi*I/*Nhe*I,

10 PCR.VII-*Bst*EII/*Nhe*I,

PCR.VIII-*Xho*I/*Eco*RI,

PCR.IX-*Bst*EII/*Nhe*I, and

PCR.X-*Pst*I/*Eco*RI

were purified on an 1.2% agarose gel using DEAE-membranes NA45 (Schleicher & Schuell) as described by Maniatis et al. The purified fragments were dissolved in H<sub>2</sub>O at a  
15 concentration of 100-150 ng/µl.

Construction of the pGOSA double head expression vectors.

The construction of pGOSA.E (see Figure 8) involved several cloning steps that produced 4 intermediate constructs

20 pGOSA.A to pGOSA.D (see Figure 30-34). The final expression vector pGOSA.E and the oligonucleotides in Table 1 above have been designed to enable most specificities to be cloned into the final pGOSA.E construct (Figure 9). The upstream V<sub>H</sub> domain can be replaced by any *Pst*I-*Bst*EII V<sub>H</sub>  
25 gene fragment obtained with oligonucleotides PCR.51 and PCR.89 (see Table 1 above). The oligonucleotides DBL.3 and DBL.4 (see Table 1 above) were designed to introduce *Sfi*I and *Nhe*I restriction sites in the V<sub>H</sub> gene fragments thus allowing cloning of those V<sub>H</sub> gene fragments into the *Sfi*I-  
30 *Nhe*I sites as the downstream V<sub>H</sub> domain. All V<sub>L</sub> gene fragments obtained with oligonucleotides PCR.116 and PCR.90 (see Table 1 above) can be cloned into the position of the V<sub>L</sub>.3418 gene fragment as a *Sac*I-*Xho*I fragment. A complication here however is the presence of an internal  
35 *Sac*I site in the V<sub>H</sub>.3418 gene fragment. Oligonucleotides DBL.8 and DBL.9 (see Table 1 above) are designed to allow cloning of V<sub>L</sub> gene fragments into the position of the

V<sub>L</sub>.4715 gene fragment as a *Sal*I-*Not*I fragment. The pGOSA.E derivatives pGOSA.V, pGOSA.S and pGOSA.T with only one or no linker sequences contain some aberrant restriction sites at the new joining points. The V<sub>H</sub>A-V<sub>H</sub>B construct without a linker lacks the 5' V<sub>H</sub>B *Sfi*I site. The V<sub>H</sub>B fragment is  
5 cloned into these constructs as a *Bst*EII/*Nhe*I fragment using oligonucleotides DBL.10 or DBL.11 and DBL.4 (see Table 1 above). The V<sub>L</sub>B-V<sub>L</sub>A construct without a linker lacks the 5' V<sub>L</sub>A *Sal*I site. The V<sub>L</sub>A fragment is cloned into these  
10 constructs as a *Xho*I/*Eco*RI fragment using oligonucleotides DBL.11 and DBL.9 (see Table 1 above).

In the following part of the description the following linkers are mentioned which are also present in the  
15 sequence listing:

the (Gly<sub>4</sub>Ser)<sub>3</sub> linker, present in SEQ ID NO: 2 as amino acids 109-123 and SEQ ID NO: 10 as amino acids 121-135, the (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker (= linkerA), present in SEQ ID NO: 12 as amino acids 121-139, and  
20 the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker (= linkerV), present in SEQ ID NO: 13 as amino acids 108-122.

#### **pGOSA.A**

This plasmid is derived from both the Fv.4715-myc construct and the scFv.4715-myc construct.  
25

An *Sfi*I restriction site was introduced between the DNA sequence encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker and the gene fragment encoding the V<sub>L</sub> of the scFv.4715-myc construct (see Figure 30). This was achieved by replacing the *Bst*EII-*Sac*I  
30 fragment of the latter construct by the fragment PCR-I *Bst*EII/*Sac*I (Figure 20) that contains an *Sfi*I site between the DNA encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker and the V<sub>L</sub>.4715 gene fragment. The introduction of the *Sfi*I site also introduced 4 additional amino acids (AlaGlySerAla) between the  
35 (Gly<sub>4</sub>Ser)<sub>3</sub> linker and V<sub>L</sub>.4715 resulting in a (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker (linkerA). The oligonucleotides used to produce PCR-I (DBL.1 and DBL.2,

see Table 1 above) were designed to match the sequence of the framework-3 region of V<sub>H</sub>.4715 and to prime at the junction of the DNA encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker and the V<sub>L</sub>.4715 gene fragment, respectively. Thus pGOSA.A can be  
5 indicated as: *pelB-V<sub>H</sub>4715-linkerA-(SfiI)-V<sub>L</sub>4715-myc.*

#### pGOSA.B

This plasmid is derived from plasmid Fv.3418 (see Figure 31). The XhoI-EcoRI fragment of plasmid Fv.3418 comprising  
10 the 3' end of DNA encoding framework-4 of the V<sub>L</sub> including the stop codon was removed and replaced by the fragment PCR-IV XhoI/EcoRI (Figure 23). The oligonucleotides used to produce PCR-IV (DBL.6 and DBL.7, see Table 1 above) were designed to match the sequence at the junction of the V<sub>L</sub> and  
15 the (Gly<sub>4</sub>Ser)<sub>3</sub> linker perfectly (DBL.6), and to be able to prime at the junction of the (Gly<sub>4</sub>Ser)<sub>3</sub> linker and the V<sub>H</sub> in pUR.4124 (DBL.7). DBL.7 removed the PstI site in the V<sub>H</sub> (silent mutation) and introduced a SalI restriction site at the junction of the (Gly<sub>4</sub>Ser)<sub>3</sub> linker and the V<sub>H</sub>, thereby  
20 replacing the last Ser of the linker by a Val residue resulting in a (Gly<sub>4</sub>Ser)<sub>3</sub>Gly<sub>4</sub>Val linker (linkerV). Thus pGOSA.B can be indicated as:

*pelB-V<sub>H</sub>3418 + pelB-V<sub>L</sub>3418-linkerV-(SalI-EcoRI)*

#### 25 pGOSA.C

This plasmid contains DNA encoding V<sub>H</sub>.4715 linked by the (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker to V<sub>H</sub>.3418 (see Figure 32), thus:  
*pelB-V<sub>H</sub>4715-linkerA-V<sub>H</sub>3418.*

This construct was obtained by replacing the SfiI-EcoRI  
30 fragment from pGOSA.A encoding V<sub>L</sub>.4715 by the fragment PCR-II SfiI/EcoRI containing the V<sub>H</sub>.3418 gene (see Figure 21). The oligonucleotides used to produce PCR-II (DBL.3 and DBL.4, see Table 1 above) hybridize in the framework-1 and framework-4 region of the gene encoding V<sub>H</sub>.3418,  
35 respectively. DBL.3 was designed to remove the PstI restriction site (silent mutation) and to introduce an SfiI restriction site upstream of the V<sub>H</sub> gene. DBL.4 destroys

the *Bst*EII restriction site in the framework-4 region and introduces an *Nhe*I restriction site downstream of the stopcodon.

#### 5 pGOSA.D

This plasmid contains a dicistronic operon comprising the *V<sub>H</sub>*.3418 gene and DNA encoding *V<sub>L</sub>*.3418 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to *V<sub>L</sub>*.4715 (see Figure 33), thus:

***pelB-V<sub>H</sub>3418 + pelB-V<sub>L</sub>3418-linkerV-V<sub>L</sub>4715.***

- 10 This construct was obtained by digesting plasmid pGOSA.B with *Sal*I-*Eco*RI and inserting the fragment PCR-V *Sal*I/*Eco*RI (Figure 24) containing the *V<sub>L</sub>*.4715 gene. The oligonucleotides used to obtain PCR-V (DBL.8 and DBL.9, see Table 1 above) were designed to match the nucleotide
- 15 sequence of the framework-1 and framework-4 regions of the *V<sub>L</sub>*.4715 gene, respectively. DBL.8 removed the *Sac*I site from the framework-1 region (silent mutation) and introduced a *Sal*I restriction site upstream of the *V<sub>L</sub>*.4715 gene. DBL.9 destroyed the *Xho*I restriction site in the framework-4
- 20 region of the *V<sub>L</sub>*.4715 gene (silent mutation) and introduced a *Not*I and an *Eco*RI restriction site downstream of the stop codon.

#### pGOSA.E

- 25 This plasmid contains a dicistronic operon comprising DNA encoding *V<sub>H</sub>*.4715 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>AlaGlySerAla linker to *V<sub>H</sub>*.3418 plus DNA encoding *V<sub>L</sub>*.3418 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to *V<sub>L</sub>*.4715 (see Figure 34), thus:
- pelB-V<sub>H</sub>4715-linkerA-V<sub>H</sub>3418 + pelB-V<sub>L</sub>3418-linkerV-V<sub>L</sub>4715.***
- 30 Both translational units are preceded by a ribosome binding site and DNA encoding a *pelB* leader sequence. This plasmid was obtained by a three-point ligation by mixing the vector resulting from pGOSA.D after removal of the *V<sub>H</sub>*3418-encoding *Pst*I-*Sac*I insert with the *Pst*I-*Nhe*I pGOSA.C insert
- 35 containing *V<sub>H</sub>*.4715 linked to *V<sub>H</sub>*.3418 and the PCR-III *Nhe*I/*Sac*I fragment (see Figure 22). The remaining *Pst*I-*Sac*I pGOSA.D vector contains the 5' end of the framework-1

region of V<sub>H</sub>.3418 upto the *Pst*I restriction site and V<sub>L</sub>.3418 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to V<sub>L</sub>.4715 starting from the *Sac*I restriction site in V<sub>L</sub>.3418. The *Pst*I-*Nhe*I pGOSA.C insert contains V<sub>H</sub>.4715 linked by the (Gly<sub>4</sub>Ser)<sub>3</sub>-  
5 AlaGlySerAla linker to V<sub>H</sub>.3418, starting from the *Pst*I restriction site in the framework-1 region in V<sub>H</sub>.4715. The *Nhe*I-*Sac*I PCR-III fragment provides the ribosome binding site and DNA encoding the *pelB* leader sequence for the V<sub>L</sub>.3418-(Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val-V<sub>L</sub>.4715 construct. The oligo-  
10 nucleotides DBL.5 and PCR.116 (see Table 1 above) used to generate PCR-III were designed to match the sequence upstream of the ribosome binding site of V<sub>L</sub>.4715 in Fv.4715 and to introduce an *Nhe*I restriction site (DBL.5), and to match the framework-4 region of V<sub>L</sub>.3418 (PCR.116).

15

**pGOSA.V**

This plasmid is derived from pGOSA.E (see Figure 35) from which the *Bst*EII/*Nhe*I fragment containing DNA encoding linkerA-V<sub>H</sub>.3418 was excised and replaced by the fragment  
20 PCR-VII *Bst*EII/*Nhe*I containing the V<sub>H</sub>.3418 gene (see Figure 26). The resulting plasmid pGOSA.V contains V<sub>H</sub>.3418 linked directly to the framework-4 region of V<sub>H</sub>.4715, plus V<sub>L</sub>.4715 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to the framework-4 region of V<sub>L</sub>.3418, thus:

25 ***pelB*-V<sub>H</sub>4715\*V<sub>H</sub>3418 + *pelB*-V<sub>L</sub>3418-linkerV-V<sub>L</sub>4715.**

**pGOSA.S**

This plasmid is derived from pGOSA.E (see Figure 36) from which the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val-V<sub>L</sub>4715 *Xho*I/*Eco*RI fragment was  
30 excised and replaced by the fragment PCR-VIII *Xho*I/*Eco*RI which contains V<sub>L</sub>.4715 (see Figure 27). The resulting plasmid pGOSA.S contains V<sub>H</sub>.4715 linked by the (Gly<sub>4</sub>Ser)<sub>3</sub>-AlaGlySerAla linker to V<sub>H</sub>.3418 plus V<sub>L</sub>.3418 linked directly to the 5' end of the framework-1 region of V<sub>L</sub>.4715, thus:

35 ***pelB*-V<sub>H</sub>.4715-linkerA-V<sub>H</sub>.3418 + *pelB*-V<sub>L</sub>.3418\*V<sub>L</sub>.4715.**

**pGOSA.T**

This plasmid contains a dicistronic operon consisting of  $V_H.3418$  directly to the framework-4 region of  $V_H.4715$  plus  $V_L.3418$  linked directly to the 5' end of the framework-1 region of  $V_L.4715$  (see Figure 37). Both transcriptional units are preceded by a ribosome binding site and a *pelB* leader sequence, thus:

$pelB-V_H.4715*V_H.3418 + pelB-V_L.3418*V_L.4715.$

This construct was obtained by inserting the *NheI/EcoRI* fragment of pGOSA.S which contains  $V_L.3418$  linked directly to the 5' end of the framework-1 region of  $V_L.4715$ , into the vector pGOSA.V from which the *NheI/EcoRI* fragment containing  $V_L.3418$  linked by the  $(Gly_4Ser)_2Gly_4Val$  linker to  $V_L.4715$  was removed.

15

**EXAMPLE 6.** Detailed description of the preparation of other dicistronic constructs pGOSA.G, and pGOSA.J, pGOSA.Z, pGOSA.AA and pGOSA.AB

20

**pGOSA.G**

This plasmid is an intermediate for the synthesis of pGOSA.J. It is derived from pGOSA.E from which the  $V_H.4715$  *PstI/BstEII* fragment has been excised and replaced by the  $V_H.3418$  *PstI/BstEII* fragment (excised from Fv.3418). The resulting plasmid pGOSA.G (see Figure 38) contains two copies of  $V_H.3418$  linked by the  $(Gly_4Ser)_2AlaGlySerAla$  linker, plus  $V_L.4715$  linked by the  $(Gly_4Ser)_2Gly_4Val$  linker to the framework-4 region of  $V_L.3418$ , thus:

$pelB-V_H.3418-linkerA-V_H.3418 + pelB-V_L.3418-linkerV-V_L.4715.$

**pGOSA.J**

This plasmid contains a dicistronic operon consisting of  $V_H.3418$  linked by the  $(Gly_4Ser)_2AlaGlySerAla$  linker to  $V_H.4715$  plus  $V_L.3418$  linked by the  $(Gly_4Ser)_2Gly_4Val$  linker to  $V_L.4715$ . Both transcriptional units are preceded by a

ribosome binding site and a *pelB* leader sequence (see Figure 39), thus:

*pelB-V<sub>H</sub>.3418-linkerA-V<sub>H</sub>.4715 + pelB-V<sub>L</sub>.3418-linkerV-V<sub>L</sub>.4715.*

- 5 This construct was obtained by inserting the fragment PCR-VI *SfiI/NheI* which contains V<sub>H</sub>4715 (Figure 25), into the vector pGOSA.G from which the *SfiI/NheI* V<sub>H</sub>3418 fragment was removed.

10 **pGOSA.Z**

This plasmid is derived from pGOSA.G from which the (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker-V<sub>H</sub>3418 *BstEII/NheI* fragment was excised and replaced by the fragment PCR-IX *BstEII/NheI* which contains V<sub>H</sub>.4715 (Figure 28). The resulting plasmid  
15 pGOSA.Z (see Figure 40) contains V<sub>H</sub>.3418 linked directly to the framework-1 region of V<sub>H</sub>.4715, plus V<sub>L</sub>.4715 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to the framework-4 region of V<sub>L</sub>.3418, thus:

*pelB-V<sub>H</sub>.3418\*V<sub>H</sub>.4715 + pelB-V<sub>L</sub>.3418-linkerV-V<sub>L</sub>.4715.*

20

**pGOSA.AA**

- This plasmid contains a dicistronic operon consisting of the V<sub>H</sub>.3418 linked directly to the 5' end of the framework-1 region of V<sub>H</sub>.4715 plus V<sub>L</sub>.3418 linked directly to the 5'  
25 end of the framework-1 region of V<sub>L</sub>.4715. Both transcriptional units are preceded by a ribosome binding site and a *pelB* leader sequence (see Figure 41). This construct was obtained by inserting the *NheI/EcoRI* fragment of pGOSA.T which contains V<sub>L</sub>.3418 linked directly to the 5'  
30 end of the framework-1 region of V<sub>L</sub>.4715, into the vector pGOSA.Z from which the *NheI/EcoRI* fragment containing V<sub>L</sub>.3418 linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to V<sub>L</sub>.4715 was removed, thus:

*pelB-V<sub>H</sub>.3418\*V<sub>H</sub>.4715 + pelB-V<sub>L</sub>.3418\*V<sub>L</sub>.4715.*

35



**pGOSA.AB**

This plasmid is derived from pGOSA.J by a three point ligation reaction (see Figure 42). The *SacI*/*EcoRI* insert, containing part of  $V_H.3418$  and the full  
5 (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker- $V_H.4715$  and the  $V_L.3418$ -  
(Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val- $V_L.4715$  encoding sequences, was removed and replaced by the *SacI*/*SacI* pGOSA.J fragment containing the same part of  $V_H.3418$  and the full (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker- $V_H.4715$  and the *SacI*/*EcoRI* pGOSA.T fragment  
10 containing  $V_L.3418$  linked directly to the framework-1 region of  $V_L.4715$  (see Figure 37). The resulting plasmid contains  $V_H.3418$  linked by the (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla linker to the 5' end of the framework-1 region of  $V_H.4715$  plus  $V_L.3418$  linked directly to the 5' end of the framework-1 region of  
15  $V_L.4715$ , thus:

$pelB-V_H.3418$ -linkerA- $V_H.4715$  +  $pelB-V_L.3418 * V_L.4715$ .

20 **EXAMPLE 7.** Detailed description of the preparation of monocistronic constructs pGOSA.L and pGOSA.Y, and pGOSA.C, pGOSA.X, pGOSA.AC and pGOSA.AD

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**pGOSA.L**

This plasmid is derived from pGOSA.E from which the  
25 *HindIII*/*NheI* fragment containing DNA encoding  $V_H.4715$ -  
(Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla- $V_H.3418$  was removed (see Figure 43). The DNA ends of the vector were made blunt-end using Klenow DNA polymerase and ligated. The resulting plasmid pGOSA.L contains  $V_L.3418$  linked by the (Gly<sub>4</sub>Ser)<sub>2</sub>Gly<sub>4</sub>Val linker to  
30 the 5' end of the framework-1 region of  $V_L.4715$ , thus:

$pelB-V_L.3418$ -linkerV- $V_L.4715$ .

**pGOSA.Y**

This plasmid is derived from pGOSA.T from which the  
35 *HindIII*/*NheI* fragment containing DNA encoding  $V_H.4715$ -  
 $V_H.3418$  was removed (see Figure 44). The DNA ends of the vector were made blunt-end using Klenow DNA polymerase and

ligated. The resulting plasmid pGOSA.Y contains  $V_L.3418$  linked directly to 5' end of the framework-1 region of  $V_L.4715$ , thus:

**$pelB-V_L.3418*V_L.4715$ .**

5

The preparation of pGOSA.C was given in Example 5 above; it can be indicated with:  **$pelB-V_H.4715-linkerA-V_H.3418$ .**

#### **pGOSA.X**

10 This plasmid is derived from pGOSA.T from which the *NheI/EcoRI* fragment containing DNA encoding  $V_L.3418-V_L.4715$  was removed. The DNA ends of the vector were made blunt-end using Klenow DNA polymerase and ligated. The resulting plasmid pGOSA.X (see Figure 45) contains  $V_H.4715$  linked  
15 directly to 5' end of the framework-1 region of  $V_H.3418$ , thus:

**$pelB-V_H.4715*V_H.3418$ .**

#### **pGOSA.AC**

This plasmid is derived from pGOSA.Z from which the  
20 *NheI/EcoRI* fragment containing DNA encoding  $V_L.3418-(Gly_4Ser)_2Gly_4Val-V_L.4715$  was removed (see Figure 46). The DNA ends of the vector were made blunt-end using Klenow DNA polymerase and ligated. The resulting plasmid pGOSA.AC contains  $V_H.3418$  linked directly to 5' end of the  
25 framework-1 region of  $V_H.4715$ , thus:

**$pelB-V_H.3418*V_H.4715$ .**

#### **pGOSA.AD**

This plasmid was obtained by inserting the *PstI/EcoRI* PCR.X  
30 fragment containing DNA encoding  $V_H.3418-(Gly_4Ser)_3AlaGly-SerAla-V_H.4715$  (see Figure 29) into the Fv.4715-myc vector from which the *PstI/EcoRI* Fv.4715-myc insert was removed (see Figure 47), thus:  **$pelB-V_H.3418-linkerA-V_H.4715$ .**

35 These monocistronic constructs can be used to transform the same host with two different plasmids or to transform two

different hosts, so that the two  $V_H$ 's in series can be produced separately from the two  $V_L$ 's in series.

#### Evaluation of the results obtained

##### 5 Bifunctional binding activity of GOSA double heads

In this specification the construction of a two chain protein complex is described, in which one of the chains consists of two heavy chain V-domains and the other chain consists of the two corresponding light chain V-domains.

- 10 The variable domains are linked either directly or through a polypeptide linker. In this specification evidence is provided that these type of molecules ("double heads") contain both antigen binding specificities of the Fv's used to generate these multi-functional antibody fragments.
- 15 Figure 12 shows that GOSA.E can be used to specifically target the enzyme glucose oxidase to several *Streptococcus sanguis* strains, using antibody fragments derived from hybridomas expressing antibodies directed against these antigens. Figure 12 further shows that the fine specificity
- 20 of the anti-*Streptococcus sanguis* scFv 4715 is preserved in the GOSA.E double head.

#### **Effect of linkers and relative position of V-domains on double head activity**

- 25 After it was shown that the "cross-over double-head" approach ( $V_HA-V_HB + V_LB-V_LA$ ) yields active bispecific molecules, the importance of the relative position of the V-domains in these constructs was investigated. Both possible positional orientations ( $GOSA.E = V_HA-LinkerA-V_HB +$
- 30  $V_LB-LinkerV-V_LA$  and  $GOSA.J = V_HB-LinkerA-V_HA + V_LB-LinkerV-V_LA$ ) were constructed and tested for bispecific activity, despite the suggestion obtained by molecular modelling that the binding site formed by the second (downstream/C-terminal) V-domains in the configuration  $V_HB-V_HA + V_LB-V_LA$
- 35 ( $GOSA.J$ ) was in an unfavourable position for binding to large protein antigens on the surface of cells. Surprisingly however, it was found experimentally that the

- downstream binding site is in fact accessible. Although the relative position of the heavy chains and the light chains was found to have an effect on the observed reactivity both tested combinations show bispecific activity with the
- 5 "cross-over" combination ( $GOSA.E = V_HA-V_HB + V_LB-V_LA$ ) exhibiting a higher level of reactivity compared to the combination  $V_HB-V_HA + V_LB-V_LA$  ( $= GOSA.J$ ) as demonstrated for A=anti-Strep and B=anti-Gox.
- 10 Molecular modelling of the  $V_HB-V_HA + V_LB-V_LA$  ( $= GOSA.J$ ) configuration further suggested that, only when the connecting linkers are kept long enough (to span 30 to 35 Å), the protein chains could fold such that both binding sites are fully accessible.
- 15 The "cross-over" configuration:  $V_HA-V_HB + V_LB-V_LA$  ( $GOSA.E$ ) wherein linker length was not critical, was predicted to result in a complex with both binding sites facing in opposite directions, without the restraints suggested for the configuration  $V_HB-V_HA + V_LB-V_LA$  ( $GOSA.J$ ).
- 20 Removing the flexible polypeptide linker from the  $V_HA-V_HB$  chain only had a minimal effect on the ability of the double head in the "cross-over" configuration ( $GOSA.V = V_HA*V_HB + V_LB-V_LA$ ) to bind both *S. sanguis* and Glucose oxidase. However, removing the flexible polypeptide linker
- 25 from the  $V_HB-V_HA$  chain from the molecule in the  $V_HB-V_HA + V_LB-V_LA$  configuration ( $GOSA.Z = V_HB*V_HA + V_LB-V_LA$ ) resulted in a dramatic reduction of its ability to bind both *S. sanguis* and Glucose oxidase.
- In contrast with the double head in the "cross-over"
- 30 configuration without the flexible polypeptide linker between the two heavy chain domains ( $GOSA.V$ ), where molecular modelling predicted the resulting molecule to be active, removal of the flexible linker from the  $V_LB-V_LA$  chain could not be modelled such that both binding sites
- 35 were fully accessible. ELISA results confirm that the double head in the  $V_HB-V_HA + V_LB-V_LA$  configuration without a linker between the two light chain domains ( $GOSA.AB$ )

exhibits only minimal *S. sanguis* and glucose oxidase binding activity. Surprisingly, deletion of the flexible linker from the  $V_L B-V_L A$  chain from the double head in the "cross-over" configuration (GOSA.S) only had a small effect  
5 on the bispecific activity of the resulting molecule. As expected from the molecular modelling results from the double heads without a flexible linker between the two light chain domains, removal of both the flexible polypeptide linkers from the double head molecules, could  
10 not be modelled such that both binding sites were fully accessible. In agreement with the ELISA results obtained with the GOSA.AB construct, the double head in the  $V_H B-V_H A + V_L B-V_L A$  configuration without any linkers (GOSA.AA) only exhibits minimal if any *S. sanguis* and glucose oxidase  
15 binding activity. Surprisingly, the double head in the "cross-over" configuration without any linkers (GOSA.T =  $V_H A * V_H B + V_L B * V_L A$ ) still exhibited 25-50% of *S. sanguis* and glucose oxidase bispecific binding activity when compared to the activity of the double head in the "cross-over"  
20 configuration with two linkers (GOSA.E). Thus the conclusion of this work is that modelling can give some indications, but that the computer programmes cannot predict what is possible and what not. Several deviations from the modelling expectations were found. With a  
25 paraphrase on an old saying: theories are nice but the experiment is the ultimate proof.

#### **Sensitivity of GOSA double heads**

Using an ELISA format it was shown that the sensitivity of  
30 the GOSA.E double head is as least as a sensitive as an IgG-glucose oxidase conjugate, as determined by the lowest concentration of *Streptococcus sanguis* antigen immobilised on a solid phase that is still detectable.

#### **35 GOSA double heads are produced as dimers**

FPLC analysis of partially affinity-purified GOSA.E, GOSA.V, GOSA.S and GOSA.T samples usually gave only one

GOSA double head activity peak as determined by ELISA (Figures 16-19). The position of this peak in the elution pattern indicated that the molecular weight of the GOSA double head is 40-50 kD. Since this molecular weight corresponds to the expected molecular weight of the  $V_H2 + V_L2$  double head dimer, it was concluded that GOSA.E, GOSA.V, GOSA.S and GOSA.T are primarily produced as dimeric molecules. Occasionally an activity peak with an apparent molecular weight of  $\approx 200$  kD was observed (Figure 16). The presence of glucose oxidase activity in these fractions indicate that these fractions contain GOSA double head complexed with glucose oxidase.

#### In vitro assembly of GOSA double heads

It was shown that bifunctionally active dimeric GOSA molecules together in one cell can be produced by translation from one dicistronic messenger (GOSA.E, GOSA.S, GOSA.T, GOSA.V, GOSA.J, GOSA.AB, GOSA.AA and GOSA.Z). In addition high levels of *S. sanguis* and glucose oxidase bispecific binding activity is formed when supernatants of cultures producing the separate GOSA subunits are mixed (see Example 7). The effects of linkers and the relative position of the individual  $V_H$ -domains on the *S. sanguis* and glucose oxidase bispecific binding activity observed in these mixing experiments are comparable to the dicistronic constructs.

The constructs described above are summarised in Table 2 below.

Table 2A describes intermediate constructs that were not further tested.

Table 2B describes the dicistronic constructs.

Table 2C describes the monocistronic constructs.

(LiA) stands for the  $V_H-V_H$  linker (Gly<sub>4</sub>Ser)<sub>3</sub>AlaGlySerAla  
(= linkerA)  
(LiV) stands for the  $V_L-V_L$  linker (Gly<sub>4</sub>Ser)<sub>3</sub>Gly<sub>4</sub>Val  
(= linkerV)

(\*) indicates that the two heavy chain domains or the two light chain domains are fused together without a connecting linker.

5

Table 2.

Table 2A

	GOSA.A :	V <sub>H</sub> .4715-LiA-( <i>Sfi</i> I)-V <sub>L</sub> .4715-myc
10	GOSA.B :	V <sub>H</sub> .3418-LiV-V <sub>L</sub> .3418-( <i>Sal</i> I/ <i>Eco</i> RI)
	GOSA.D :	V <sub>H</sub> .3418 + V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715
	GOSA.G :	V <sub>H</sub> .3418-LiA-V <sub>H</sub> .3418 + V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715

Table 2B

15	GOSA.E :	V <sub>H</sub> .4715-LiA-V <sub>H</sub> .3418 + V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715
	GOSA.S :	V <sub>H</sub> .4715-LiA-V <sub>H</sub> .3418 + V <sub>L</sub> .3418*V <sub>L</sub> .4715
	GOSA.T :	V <sub>H</sub> .4715*V <sub>H</sub> .3418 + V <sub>L</sub> .3418*V <sub>L</sub> .4715
	GOSA.V :	V <sub>H</sub> .4715*V <sub>H</sub> .3418 + V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715
20	GOSA.J :	V <sub>H</sub> .3418-LiA-V <sub>H</sub> .4715 + V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715
	GOSA.AB:	V <sub>H</sub> .3418-LiA-V <sub>H</sub> .4715 + V <sub>L</sub> .3418*V <sub>L</sub> .4715
	GOSA.AA:	V <sub>H</sub> .3418*V <sub>H</sub> .4715 + V <sub>L</sub> .3418*V <sub>L</sub> .4715
	GOSA.Z :	V <sub>H</sub> .3418*V <sub>H</sub> .4715 + V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715

25 Table 2C

	GOSA.L :	V <sub>L</sub> .3418-LiV-V <sub>L</sub> .4715
	GOSA.Y :	V <sub>L</sub> .3418*V <sub>L</sub> .4715
	GOSA.AD:	V <sub>H</sub> .3418-LiA-V <sub>H</sub> .4715
30	GOSA.AC:	V <sub>H</sub> .3418*V <sub>H</sub> .4715
	GOSA.C :	V <sub>H</sub> .4715-LiA-V <sub>H</sub> .3418
	GOSA.X :	V <sub>H</sub> .4715*V <sub>H</sub> .3418

**REFERENCES****Patent literature**

- EP-0281604; GENEX/ENZON; SINGLE POLYPEPTIDE CHAIN BINDING MOLECULES; priority date 02.09.86.
- 5 - WO 93/11161; ENZON, INC; MULTIVALENT ANTIGEN-BINDING PROTEINS; priority date 25.11.91.
- WO 94/09131; SCOTGEN LIMITED; RECOMBINANT SPECIFIC BINDING PROTEIN; priority date 15.10.92).
- WO 94/13804 (CAMBRIDGE ANTIBODY TECHNOLOGY / MEDICAL
- 10 RESEARCH COUNCIL; MULTIVALENT AND MULTISPECIFIC BINDING PROTEINS, THEIR MANUFACTURE AND USE; first priority date 04.12.92
- WO 94/13806; THE DOW CHEMICAL COMPANY; MULTIVALENT SINGLE CHAIN ANTIBODIES; priority date 11.12.92
- 15 - WO 94/25591; UNILEVER N.V., UNILEVER PLC, Hamers, R., Hamers-Casterman, C., & Muyldermans, S; PRODUCTION OF ANTIBODIES OR (FUNCTIONALIZED) FRAGMENTS THEREOF DERIVED FROM HEAVY CHAIN IMMUNOGLOBULINS OF CAMELIDAE; first

20

**Non-patent literature**

- Anthony, J., Near, R., Wong, S.L., Iida, E., Ernst, E., Wittekind, M., Haber, E. and Ng, S-C; *Molec. Immunol.* 29 (1992) 1237-1247, PRODUCTION OF STABLE ANTI-DIGOXIN Fv IN
- 25 *ESCHERICHIA COLI*.
- Berry, M.J. and Davies, J.; *J. Chromatography* 597 (1992) 239-245; Use of antibody fragments in immunoaffinity chromatography: comparison of Fv fragments, V<sub>H</sub> fragments and paralog peptides.
- 30 - Better, M., Chang, C.P., Robinson, R.R. and Horwitz A.H.; *Science* 240 (1988) 1041-1043; *Escherichia coli* Secretion of an Active Chimeric Antibody Fragment.
- Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan,
- 35 G.S. and Whitlow, M.; *Science* 242 (1988) 423-426; Single-Chain Antigen-Binding Proteins.



- Carter, P., Kelley, R.F., Rodrigues, M.L., Snedecor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourell, J.H., Shepard, H.M. and Henner, D.; **BIO/TECHNOLOGY** 10 (1992) 163-167; High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment.
- Cumber, A.J., Ward, E.S., Winter, G., Parnell, G.D. and Wawrzynczak, E.J.; **J. Immunol.** 149 (1992) 120-126; COMPARATIVE STABILITIES IN VITRO AND IN VIVO OF A RECOMBINANT MOUSE ANTIBODY FvCys FRAGMENT AND A bisFvCys CONJUGATE.
- Firek, S., Draper, J., Owen, M.R.L., Gandecha, A., Cockburn, B., and Whitlam, G.C.; **Plant Mol. Biol.** 23 (1993) 861-870; Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures.
- Givol, D.; **Molec. Immunol.** 28 (1991) 1379; THE MINIMAL ANTIGEN BINDING FRAGMENT OF ANTIBODIES - Fv FRAGMENT.
- Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Bajyana Songa, E., Bendahman, N. and Hamers, R.; **Nature** 363 (1993) 446-448; Naturally occurring antibodies devoid of light chains.
- Hiatt, A., Cafferkey, R. and Bowdish, K.; **Nature** 342 (1989) 76-78; Production of antibodies in transgenic plants.
- Holliger, P., Prospero, T., and Winter, G.; **Proc. Natl. Acad. Sci. USA** 90 (1993) 6444-6448; "Diabodies": Small bivalent and bispecific antibody fragments.
- Horwitz, A.H., Chang, C.P., Better, M., Hellstrom, K.E. and Robinson, R.R.; **Proc. Natl. Acad. Sci. USA** 85 (1988) 8678-8682; Secretion of functional antibody and Fab fragment from yeast cells.
- Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M-S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and Oppermann, H.; **Proc. Natl. Acad. Sci. USA** 85 (1988) 5879-5883; Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*.

- Kostelny, S.A., Cole, M.S. and Tso, J.Y.; *J. Immunol.* 148 (1992) 1547-1553; FORMATION OF A BISPECIFIC ANTIBODY BY THE USE OF LEUCINE ZIPPERS.
- Mallender, W.D., and Voss, Jr., E.W.; *J. Biol. Chem.* 269 5 (1994) 199-206; Construction, Expression, and Activity of a Bivalent Bispecific Single-chain Antibody.
- Milstein, C. and Cuello, A.C.; *Nature*, 305 (1983) 537-540; Hybrid hybridomas and their use in immunohistochemistry.
- 10 - Nyyssönen, E., Penttillä, M., Harkki, A., Saloheimo, A., Knowles, J.K.C. and Keränen, S.; *BIO/TECHNOLOGY Bio/tech.* 11 (1993) 591-595; Efficient Production of Antibody Fragments in Filamentous Fungus *Trichoderma reesei*.
- Owen, M., Gandeche, A., Cockburn, B. and Whitelam G.;  
15 *BIO/TECHNOLOGY* 10 (1992) 790-794; Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco.
- Pack, P. and Plückthun, A.; *Biochemistry* 31 (1992) 1579-1584; Miniantibodies: Use of Amphipathic Helices to Produce  
20 Functional, Flexibly Linked Dimeric Fv Fragments with High Avidity in *Escherichia coli*.
- Skerra, A. and Plückthun, A.; *Science*, 240 (1988) 1038-1041; Assembly of a Functional Immunoglobulin Fv Fragment in *Escherichia coli*.
- 25 - Taub, R., Gould, R.J., Ciccarone, T.M., Hoxie, J., Friedman, P.A., Shattil, S.J. and Garsky, V.M.; *J. Biol. Chem.* 264 (1989) 259-265; A Monoclonal Antibody against the Platelet Fibrinogen Receptor Contains a Sequence That Mimics a Receptor Recognition Domain in Fibrinogen.
- 30 - Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. and Winter G.; *Nature* 341 (1989) 544-546; Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*.
- Williams, W.V., Moss, D.A., Kieber-Emmons, T., Cohen,  
35 J.A., Myers, J.N., Weiner, D.B. and Greene, M.I.; *Proc. Natl. Acad. Sci. USA.* 86 (1989) 5537-5541; Development of biologically active peptides based on antibody structure.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: Unilever PLC  
(B) STREET: Unilever House, Blackfriars  
(C) CITY: London  
(E) COUNTRY: United Kingdom  
(F) POSTAL CODE (ZIP): EC4P 4BQ (GB)

(A) NAME: Unilever N.V.  
(B) STREET: Weena 455  
(C) CITY: Rotterdam  
(E) COUNTRY: The Netherlands  
(F) POSTAL CODE (ZIP): NL-3013 AL

(A) NAME: Paul James DAVIS  
(B) STREET: The Hawthorns, Pavenham Road  
(C) CITY: Felmersham (Bedfordshire)  
(E) COUNTRY: United Kingdom  
(F) POSTAL CODE (ZIP): MK43 7EX (GB)

(A) NAME: Cornelis Paul Erik van der LOGT  
(B) STREET: 1 Bluebell Rise (Peverel Manor Estate)  
(C) CITY: Rushden (Northamptonshire)  
(E) COUNTRY: United Kingdom  
(F) POSTAL CODE (ZIP): NN10 OTU (GB)

(A) NAME: Martine Elisa VERHOEIJEN  
(B) STREET: 1 Tintagel Close (Manor Farm Estate)  
(C) CITY: Rushden (Northamptonshire)  
(E) COUNTRY: United Kingdom  
(F) POSTAL CODE (ZIP): NN10 ONP (GB)

(A) NAME: Steve Wilson  
(B) STREET: 3 Aldenham Close (Goldington )  
(C) CITY: Bedford,  
(E) COUNTRY: United Kingdom  
(F) POSTAL CODE (ZIP): MK41 0FQ (GB)

(ii) TITLE OF INVENTION: A bifunctional or bivalent  
antibody fragment analogue

(iii) NUMBER OF SEQUENCES: 31

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 95307332.7

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 737 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "cDNA domains with synthetic linker(s)"

## (vii) IMMEDIATE SOURCE:

(B) CLONE: EcoRI-HindIII insert of pUR4124

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:11..730

(D) OTHER INFORMATION:/product= "VLlys-GS-VHlys"

## (ix) FEATURE:

(A) NAME/KEY: mat\_peptide

(B) LOCATION:11..334

(D) OTHER INFORMATION:/product= "VLlys"

## (ix) FEATURE:

(A) NAME/KEY: misc\_RNA

(B) LOCATION:335..379

(D) OTHER INFORMATION:/product= "(Gly4Ser)3 linker"

## (ix) FEATURE:

(A) NAME/KEY: mat\_peptide

(B) LOCATION:380..727

(D) OTHER INFORMATION:/product= "VHlys"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCGGCC	GAC	ATC	GAG	CTC	ACC	CAG	TCT	CCA	GCC	TCC	CTT	TCT	GCG	49
	Asp	Ile	Glu	Leu	Thr	Gln	Ser	Pro	Ala	Ser	Leu	Ser	Ala	
	1				5					10				
TCT	GTG	GGA	GAA	ACT	GTC	ACC	ATC	ACA	TGT	CGA	GCA	AGT	GGG	97
Ser	Val	Gly	Glu	Thr	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gly	
	15				20					25				
CAC	AAT	TAT	TTA	GCA	TGG	TAT	CAG	CAG	AAA	CAG	GGA	AAA	TCT	145
His	Asn	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Gln	Gly	Lys	Ser	
	30				35				40				45	
CTC	CTG	GTC	TAT	TAT	ACA	ACA	ACC	TTA	GCA	GAT	GGT	GTG	CCA	193
Leu	Leu	Val	Tyr	Tyr	Thr	Thr	Thr	Leu	Ala	Asp	Gly	Val	Pro	
			50					55				60		
TTC	AGT	GGC	AGT	GGA	TCA	GGA	ACA	CAA	TAT	TCT	CTC	AAG	ATC	241
Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Gln	Tyr	Ser	Leu	Lys	Ile	
		65					70					75		
CTG	CAA	CCT	GAA	GAT	TTT	GGG	AGT	TAT	TAC	TGT	CAA	CAT	TTT	289
Leu	Gln	Pro	Glu	Asp	Phe	Gly	Ser	Tyr	Tyr	Cys	Gln	His	Phe	
	80					85					90			
ACT	CCT	CGG	ACG	TTC	GGT	GGA	GGG	ACC	AAG	CTC	GAG	ATC	AAA	337
Thr	Pro	Arg	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	
	95				100					105				
GGA	GGC	GGT	TCA	GGC	GGA	GGT	GGC	TCT	GGC	GGT	GGC	GGA	TCG	385
Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Ser	Gln	
	110			115			120					125		
CAG	CTG	CAG	GAG	TCA	GGA	CCT	GGC	CTG	GTG	GCG	CCC	TCA	CAG	433
Gln	Leu	Gln	Glu	Ser	Gly	Pro	Gly	Leu	Val	Ala	Pro	Ser	Gln	
			130				135					140		
TCC	ATC	ACA	TGC	ACC	GTC	TCA	GGG	TTC	TCA	TTA	ACC	GGC	TAT	481
Ser	Ile	Thr	Cys	Thr	Val	Ser	Gly	Phe	Ser	Leu	Thr	Gly	Tyr	
		145				150						155		

43

AAC TGG GTT CGC CAG CCT CCA GGA AAG GGT CTG GAG TGG CTG GGA ATG	529
Asn Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu Gly Met	
160 165 170	
ATT TGG GGT GAT GGA AAC ACA GAC TAT AAT TCA GCT CTC AAA TCC AGA	577
Ile Trp Gly Asp Gly Asn Thr Asp Tyr Asn Ser Ala Leu Lys Ser Arg	
175 180 185	
CTG AGC ATC AGC AAG GAC AAC TCC AAG AGC CAA GTT TTC TTA AAA ATG	625
Leu Ser Ile Ser Lys Asp Asn Ser Lys Ser Gln Val Phe Leu Lys Met	
190 195 200 205	
AAC AGT CTG CAC ACT GAT GAC ACA GCC AGG TAC TAC TGT GCC AGA GAG	673
Asn Ser Leu His Thr Asp Asp Thr Ala Arg Tyr Tyr Cys Ala Arg Glu	
210 215 220	
AGA GAT TAT AGG CTT GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC	721
Arg Asp Tyr Arg Leu Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val	
225 230 235	
TCC TCA TGA TAAGCTT	737
Ser Ser *	
240	

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 240 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp	Ile	Glu	Leu	Thr	Gln	Ser	Pro	Ala	Ser	Leu	Ser	Ala	Ser	Val	Gly
1				5					10					15	
Glu	Thr	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gly	Asn	Ile	His	Asn	Tyr
			20					25						30	
Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Gln	Gly	Lys	Ser	Pro	Gln	Leu	Leu	Val
		35				40						45			
Tyr	Tyr	Thr	Thr	Thr	Leu	Ala	Asp	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly
	50				55					60					
Ser	Gly	Ser	Gly	Thr	Gln	Tyr	Ser	Leu	Lys	Ile	Asn	Ser	Leu	Gln	Pro
	65				70					75				80	
Glu	Asp	Phe	Gly	Ser	Tyr	Tyr	Cys	Gln	His	Phe	Trp	Ser	Thr	Pro	Arg
				85					90					95	
Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	Gly	Gly	Gly	Gly
		100					105						110		
Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gln	Val	Gln	Leu	Gln
		115				120						125			
Glu	Ser	Gly	Pro	Gly	Leu	Val	Ala	Pro	Ser	Gln	Ser	Leu	Ser	Ile	Thr
	130				135						140				
Cys	Thr	Val	Ser	Gly	Phe	Ser	Leu	Thr	Gly	Tyr	Gly	Val	Asn	Trp	Val
145					150					155				160	

44

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Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Leu Gly Met Ile Trp Gly
      165                      170                      175
Asp Gly Asn Thr Asp Tyr Asn Ser Ala Leu Lys Ser Arg Leu Ser Ile
      180                      185                      190
Ser Lys Asp Asn Ser Lys Ser Gln Val Phe Leu Lys Met Asn Ser Leu
      195                      200                      205
His Thr Asp Asp Thr Ala Arg Tyr Tyr Cys Ala Arg Glu Arg Asp Tyr
      210                      215                      220
Arg Leu Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser *
      225                      230                      235                      240

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## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 920 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "cdna domains with synthetic linker(s)"

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: HindIII-EcoRI insert Fv.3418

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 36..443
- (D) OTHER INFORMATION: /product= "pelB-VH3418"

## (ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION: 36..101
- (D) OTHER INFORMATION: /product= "pectate lyase"

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 102..440
- (D) OTHER INFORMATION: /product= "VH3418"

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 495..884
- (D) OTHER INFORMATION: /product= "pelB-VL4318"

## (ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION: 495..560
- (D) OTHER INFORMATION: /product= "pectate lyase"

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 561..881
- (D) OTHER INFORMATION: /product= "VL3418"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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AAGCTTGCAA ATTCTATTTT AAGGAGACAG TCATA ATG AAA TAC CTA TTG CCT
      Met Lys Tyr Leu Leu Pro
      -22      -20

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53

45

ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCG ATG GCC	101
Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala	
-15 -10 -5	
CAG GTG CAG CTG CAG CAG TCA GGA CCT GAG CTG GTA AAG CCT GGG GCT	149
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala	
1 5 10 15	
TCA GTG AAG ATG TCC TGC AAG GCT TCT GGA TAC ACA TTC ACT AGC TAT	197
Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr	
20 25 30	
GTT ATG CAC TGG GTG AAA CAG AAG CCT GGG CAG GGC CTT GAG TGG ATT	245
Val Met His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile	
35 40 45	
GGA TAT ATT TAT CCT TAC AAT GAT GGT ACT AAG TAC AAT GAG AAG TTC	293
Gly Tyr Ile Tyr Pro Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe	
50 55 60	
AAA GGC AAG GCC ACA CTG ACT TCA GAC AAA TCC TCC AGC ACA GCC TAC	341
Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser Thr Ala Tyr	
65 70 75 80	
ATG GAG CTC AGC AGC CTG ACC TCT GAG GAC TCT GCG GTC TAT TAC TGT	389
Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys	
85 90 95	
TCA AGA CGC TTT GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC	437
Ser Arg Arg Phe Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser	
100 105 110	
TCA TAA TAAGAGCTAT GGGAGCTTGC ATGCAAATTC TATTTCAAGG AGACAGTCAT	493
Ser *	
A ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC	539
Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu	
-22 -20 -15 -10	
GCT GCC CAA CCA GCG ATG GCC GAC ATC GAG CTC ACC CAG TCT CCA TCT	587
Ala Ala Gln Pro Ala Met Ala Asp Ile Glu Leu Thr Gln Ser Pro Ser	
-5 1 5	
TCC ATG TAT GCA TCT CTA GGA GAG AGA ATC ACT ATC ACT TGC AAG GCG	635
Ser Met Tyr Ala Ser Leu Gly Glu Arg Ile Thr Ile Thr Cys Lys Ala	
10 15 20 25	
AGT CAG GAC ATT AAT ACC TAT TTA ACC TGG TTC CAG CAG AAA CCA GGG	683
Ser Gln Asp Ile Asn Thr Tyr Leu Thr Trp Phe Gln Gln Lys Pro Gly	
30 35 40	
AAA TCT CCC AAG ACC CTG ATC TAT CGT GCA AAC AGA TTG CTA GAT GGG	731
Lys Ser Pro Lys Thr Leu Ile Tyr Arg Ala Asn Arg Leu Leu Asp Gly	
45 50 55	
GTC CCA TCA AGG TTC AGT GGC AGT GGA TCT GGG CAA GAT TAT TCT CTC	779
Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Gln Asp Tyr Ser Leu	
60 65 70	
ACC ATC AGC AGC CTG GAC TAT GAA GAT ATG GGA ATT TAT TAT TGT CTA	827
Thr Ile Ser Ser Leu Asp Tyr Glu Asp Met Gly Ile Tyr Tyr Cys Leu	
75 80 85	
CAA TAT GAT GAG TTG TAC ACG TTC GGA GGG GGG ACC AAG CTC GAG ATC	875
Gln Tyr Asp Glu Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile	
90 95 100 105	

AAA CGG TAA TAATGATCAA ACGGTATAAG GATCCAGCTC GAATTC  
Lys Arg \*

920

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 136 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala  
-22 -20 -15 -10  
Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln Ser Gly Pro Glu  
-5 1 5 10  
Leu Val Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly  
15 20 25  
Tyr Thr Phe Thr Ser Tyr Val Met His Trp Val Lys Gln Lys Pro Gly  
30 35 40  
Gln Gly Leu Glu Trp Ile Gly Tyr Ile Tyr Pro Tyr Asn Asp Gly Thr  
45 50 55  
Lys Tyr Asn Glu Lys Phe Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys  
60 65 70  
Ser Ser Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Thr Ser Glu Asp  
75 80 85 90  
Ser Ala Val Tyr Tyr Cys Ser Arg Arg Phe Asp Tyr Trp Gly Gln Gly  
95 100 105  
Thr Thr Val Thr Val Ser Ser \*

110

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 130 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala  
-22 -20 -15 -10  
Ala Gln Pro Ala Met Ala Asp Ile Glu Leu Thr Gln Ser Pro Ser Ser  
-5 1 5 10  
Met Tyr Ala Ser Leu Gly Glu Arg Ile Thr Ile Thr Cys Lys Ala Ser  
15 20 25  
Gln Asp Ile Asn Thr Tyr Leu Thr Trp Phe Gln Gln Lys Pro Gly Lys  
30 35 40



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```

Ser Pro Lys Thr Leu Ile Tyr Arg Ala Asn Arg Leu Leu Asp Gly Val
      45                      50                      55
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr
      60                      65                      70
Ile Ser Ser Leu Asp Tyr Glu Asp Met Gly Ile Tyr Tyr Cys Leu Gln
      75                      80                      85                      90
Tyr Asp Glu Leu Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
      95                      100                      105
Arg *
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## (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 999 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "cDNA domains with synthetic linker(s)"
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: HindIII-EcoRI insert of Fv.4715-myc
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION:40..468
  - (D) OTHER INFORMATION:/product= "pelB-VH4715"
- (ix) FEATURE:
  - (A) NAME/KEY: sig\_peptide
  - (B) LOCATION:40..105
  - (D) OTHER INFORMATION:/product= "pectate lyase"
- (ix) FEATURE:
  - (A) NAME/KEY: mat\_peptide
  - (B) LOCATION:106..465
  - (D) OTHER INFORMATION:/product= "VH4715"
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION:520..963
  - (D) OTHER INFORMATION:/product= "pelB-VL4715-myc"
- (ix) FEATURE:
  - (A) NAME/KEY: sig\_peptide
  - (B) LOCATION:520..585
  - (D) OTHER INFORMATION:/product= "pectate lyase"
- (ix) FEATURE:
  - (A) NAME/KEY: mat\_peptide
  - (B) LOCATION:586..927
  - (D) OTHER INFORMATION:/product= "VL4715"
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_RNA
  - (B) LOCATION:928..960
  - (D) OTHER INFORMATION:/product= "myc-tag"

48

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Met Lys Tyr Leu Leu	
-22 -20	
CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCG ATG	102
Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met	
-15 -10 -5	
GCC CAG GTG CAG CTG CAG GAG TCA GGG GGA GAC TTA GTG AAG CCT GGA	150
Ala Gln Val Gln Leu Gln Glu Ser Gly Gly Asp Leu Val Lys Pro Gly	
1 5 10 15	
GGG TCC CTG ACA CTC TCC TGT GCA ACC TCT GGA TTC ACT TTC AGT AGT	198
Gly Ser Leu Thr Leu Ser Cys Ala Thr Ser Gly Phe Thr Phe Ser Ser	
20 25 30	
TAT GCC TTT TCT TGG GTC CGC CAG ACC TCA GAC AAG AGT CTG GAG TGG	246
Tyr Ala Phe Ser Trp Val Arg Gln Thr Ser Asp Lys Ser Leu Glu Trp	
35 40 45	
GTC GCA ACC ATC AGT AGT ACT GAT ACT TAT ACC TAT TAT TCA GAC AAT	294
Val Ala Thr Ile Ser Ser Thr Asp Thr Tyr Thr Tyr Ser Asp Asn	
50 55 60	
GTG AAG GGG CGC TTC ACC ATC TCC AGA GAC AAT GGC AAG AAC ACC CTG	342
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Gly Lys Asn Thr Leu	
65 70 75	
TAC CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC ACA GCC GTG TAT TAC	390
Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr	
80 85 90 95	
TGT GCA AGA CAT GGG TAC TAT GGT AAA GGC TAT TTT GAC TAC TGG GGC	438
Cys Ala Arg His Gly Tyr Tyr Gly Lys Gly Tyr Phe Asp Tyr Trp Gly	
100 105 110	
CAA GGG ACC ACG GTC ACC GTC TCC TCA TAA TAAGAGCTAT GGGAGCTTGC	488
Gln Gly Thr Thr Val Thr Val Ser Ser *	
115 120	
ATGCAAATTC TATTTCAAGG AGACAGTCAT A ATG AAA TAC CTA TTG CCT ACG	540
Met Lys Tyr Leu Leu Pro Thr	
-22 -20	
GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCG ATG GCC GAC	588
Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala Met Ala Asp	
-15 -10 -5 1	
ATC GAG CTC ACT CAG TCT CCA TTC TCC CTG ACT GTG ACA GCA GGA GAG	636
Ile Glu Leu Thr Gln Ser Pro Phe Ser Leu Thr Val Thr Ala Gly Glu	
5 10 15	
AAG GTC ACT ATG AAT TGC AAG TCC GGT CAG AGT CTG TTA AAC AGT GTA	684
Lys Val Thr Met Asn Cys Lys Ser Gly Gln Ser Leu Leu Asn Ser Val	
20 25 30	
AAT CAG AGG AAC TAC TTG ACC TGG TAC CAG CAG AAG CCA GGG CAG CCT	732
Asn Gln Arg Asn Tyr Leu Thr Trp Tyr Gln Gln Lys Pro Gly Gln Pro	
35 40 45	
CCT AAA CTG TTG ATC TAC TGG GCA TCC ACT AGG GAA TCT GGA GTC CCT	780
Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser Gly Val Pro	
50 55 60 65	

49

GAT CGC TTC ACA GCC AGT GGA TCT GGA ACA GAT TTC ACT CTC ACC ATC	828
Asp Arg Phe Thr Ala Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile	
70 75 80	
AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TAC TGT CAG AAT GAT	876
Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Asn Asp	
85 90 95	
TAT ACT TAT CCG TTC ACG TTC GGA GGG GGG ACC AAG CTC GAG ATC AAA	924
Tyr Thr Tyr Pro Phe Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys	
100 105 110	
CGG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT TAA TAAGATCAAA	973
Arg Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn *	
115 120 125	
CGGTAATAAG GATCCAGCTC GAATTC	999

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 143 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Ala	
-22 -20 -15 -10	
Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Glu Ser Gly Gly Asp	
-5 1 5 10	
Leu Val Lys Pro Gly Gly Ser Leu Thr Leu Ser Cys Ala Thr Ser Gly	
15 20 25	
Phe Thr Phe Ser Ser Tyr Ala Phe Ser Trp Val Arg Gln Thr Ser Asp	
30 35 40	
Lys Ser Leu Glu Trp Val Ala Thr Ile Ser Ser Thr Asp Thr Tyr Thr	
45 50 55	
Tyr Tyr Ser Asp Asn Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn	
60 65 70	
Gly Lys Asn Thr Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp	
75 80 85 90	
Thr Ala Val Tyr Tyr Cys Ala Arg His Gly Tyr Tyr Gly Lys Gly Tyr	
95 100 105	
Phe Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser *	
110 115 120	

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 148 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```

Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala
-22      -20              -15              -10

Ala Gln Pro Ala Met Ala Asp Ile Glu Leu Thr Gln Ser Pro Phe Ser
  -5              1              5              10

Leu Thr Val Thr Ala Gly Glu Lys Val Thr Met Asn Cys Lys Ser Gly
              15              20              25

Gln Ser Leu Leu Asn Ser Val Asn Gln Arg Asn Tyr Leu Thr Trp Tyr
              30              35              40

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser
              45              50              55

Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Ala Ser Gly Ser Gly
  60              65              70

Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala
  75              80              85              90

Val Tyr Tyr Cys Gln Asn Asp Tyr Thr Tyr Pro Phe Thr Phe Gly Gly
              95              100              105

Gly Thr Lys Leu Glu Ile Lys Arg Glu Gln Lys Leu Ile Ser Glu Glu
              110              115              120

Asp Leu Asn *
  -      125

```

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 924 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "cDNA domains with synthetic linker(s)"

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: HindIII-EcoRI insert of scFv.4715-myc

## (ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION:40..105
- (D) OTHER INFORMATION:/product= "pectate lyase"

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION:106..465
- (D) OTHER INFORMATION:/product= "VH4715"

## (ix) FEATURE:

- (A) NAME/KEY: misc\_RNA
- (B) LOCATION:466..510
- (D) OTHER INFORMATION:/product= "(Gly4Ser)3-linker"

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION:511..852
- (D) OTHER INFORMATION:/product= "VL4715"

## (ix) FEATURE:

- (A) NAME/KEY: misc\_RNA  
 (B) LOCATION:853..885  
 (D) OTHER INFORMATION:/product= "myc-tag"

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION:40..888  
 (D) OTHER INFORMATION:/product=  
 "pelB-VH4715-(Gly4Ser)3-VL4715-myc"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AAGCTTGCAT GCAAATTCTA TTTCAAGGAG ACAGTCATA ATG AAA TAC CTA TTG	54
Met Lys Tyr Leu Leu	
-22 -20	
CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCG ATG	102
Pro Thr Ala Ala Ala Gly Leu Leu Leu Ala Ala Gln Pro Ala Met	
-15 -10 -5	
GCC CAG GTG CAG CTG CAG GAG TCA GGG GGA GAC TTA GTG AAG CCT GGA	150
Ala Gln Val Gln Leu Gln Glu Ser Gly Gly Asp Leu Val Lys Pro Gly	
1 5 10 15	
GGG TCC CTG ACA CTC TCC TGT GCA ACC TCT GGA TTC ACT TTC AGT AGT	198
Gly Ser Leu Thr Leu Ser Cys Ala Thr Ser Gly Phe Thr Phe Ser Ser	
20 25 30	
TAT GCC TTT TCT TGG GTC CGC CAG ACC TCA GAC AAG AGT CTG GAG TGG	246
Tyr Ala Phe Ser Trp Val Arg Gln Thr Ser Asp Lys Ser Leu Glu Trp	
35 40 45	
GTC GCA ACC ATC AGT AGT ACT GAT ACT TAT ACC TAT TAT TCA GAC AAT	294
Val Ala Thr Ile Ser Ser Thr Asp Thr Tyr Thr Tyr Tyr Ser Asp Asn	
50 55 60	
GTG AAG GGG CGC TTC ACC ATC TCC AGA GAC AAT GGC AAG AAC ACC CTG	342
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Gly Lys Asn Thr Leu	
65 70 75	
TAC CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC ACA GCC GTG TAT TAC	390
Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr	
80 85 90 95	
TGT GCA AGA CAT GGG TAC TAT GGT AAA GGC TAT TTT GAC TAC TGG GGC	438
Cys Ala Arg His Gly Tyr Tyr Gly Lys Gly Tyr Phe Asp Tyr Trp Gly	
100 105 110	
CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA	486
Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly	
115 120 125	
GGT GGC TCT GGC GGT GGC GGA TCG GAC ATC GAG CTC ACT CAG TCT CCA	534
Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Glu Leu Thr Gln Ser Pro	
130 135 140	
TTC TCC CTG ACT GTG ACA GCA GGA GAG AAG GTC ACT ATG AAT TGC AAG	582
Phe Ser Leu Thr Val Thr Ala Gly Glu Lys Val Thr Met Asn Cys Lys	
145 150 155	
TCC GGT CAG AGT CTG TTA AAC AGT GTA AAT CAG AGG AAC TAC TTG ACC	630
Ser Gly Gln Ser Leu Leu Asn Ser Val Asn Gln Arg Asn Tyr Leu Thr	
160 165 170 175	

TGG TAC CAG CAG AAG CCA GGG CAG CCT CCT AAA CTG TTG ATC TAC TGG	678
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp	
180 185 190	
GCA TCC ACT AGG GAA TCT GGA GTC CCT GAT CGC TTC ACA GCC AGT GGA	726
Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Ala Ser Gly	
195 200 205	
TCT GGA ACA GAT TTC ACT CTC ACC ATC AGC AGT GTG CAG GCT GAA GAC	774
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp	
210 215 220	
CTG GCA GTT TAT TAC TGT CAG AAT GAT TAT ACT TAT CCG TTC ACG TTC	822
Leu Ala Val Tyr Tyr Cys Gln Asn Asp Tyr Thr Tyr Pro Phe Thr Phe	
225 230 235	
GGA GGG GGG ACC AAG CTC GAG ATC AAA CGG GAA CAA AAA CTC ATC TCA	870
Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Glu Gln Lys Leu Ile Ser	
240 245 250 255	
GAA GAG GAT CTG AAT TAA TAAGATCAAA CGGTAATAAG GATCCAGCTC GAATTC	924
Glu Glu Asp Leu Asn *	
260	

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 283 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Ala	
-22 -20 -15 -10	
Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Glu Ser Gly Gly Asp	
-5 1 5 10	
Leu Val Lys Pro Gly Gly Ser Leu Thr Leu Ser Cys Ala Thr Ser Gly	
15 20 25	
Phe Thr Phe Ser Ser Tyr Ala Phe Ser Trp Val Arg Gln Thr Ser Asp	
30 35 40	
Lys Ser Leu Glu Trp Val Ala Thr Ile Ser Ser Thr Asp Thr Tyr Thr	
45 50 55	
Tyr Tyr Ser Asp Asn Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn	
60 65 70	
Gly Lys Asn Thr Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp	
75 80 85 90	
Thr Ala Val Tyr Tyr Cys Ala Arg His Gly Tyr Tyr Gly Lys Gly Tyr	
95 100 105	
Phe Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly	
110 115 120	
Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Glu	
125 130 135	
Leu Thr Gln Ser Pro Phe Ser Leu Thr Val Thr Ala Gly Glu Lys Val	
140 145 150	

```

Thr Met Asn Cys Lys Ser Gly Gln Ser Leu Leu Asn Ser Val Asn Gln
155                               160                               165                               170

Arg Asn Tyr Leu Thr Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys
                               175                               180                               185

Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser Gly Val Pro Asp Arg
                               190                               195                               200

Phe Thr Ala Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser
                205                               210                               215

Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Asn Asp Tyr Thr
                220                               225                               230

Tyr Pro Phe Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Glu
235                               240                               245                               250

Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn *
                255                               260

```

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1706 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## - (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "cDNA domains with synthetic linker(s)"

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: HindIII-EcoRI insert of pGOSA.E

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 40..864
- (D) OTHER INFORMATION: /product= "pelB-VH4715-LiA-VH3418"

## (ix) FEATURE:

- (A) NAME/KEY: sig\_peptide
- (B) LOCATION: 40..105
- (D) OTHER INFORMATION: /product= "pectate lyase"

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 106..465
- (D) OTHER INFORMATION: /product= "VH4715"

## (ix) FEATURE:

- (A) NAME/KEY: misc\_RNA
- (B) LOCATION: 466..522
- (D) OTHER INFORMATION: /product= "linkerA  
(Gly4Ser)3AlaGlySerAla"

## (ix) FEATURE:

- (A) NAME/KEY: mat\_peptide
- (B) LOCATION: 523..861
- (D) OTHER INFORMATION: /product= "VH3418"

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 913..1689
- (D) OTHER INFORMATION: /product= "pelB-VL3418-LiV-VL4715"

(ix) FEATURE:  
 (A) NAME/KEY: sig\_peptide  
 (B) LOCATION:913..978  
 (D) OTHER INFORMATION:/product= "pectate lyase"

(ix) FEATURE:  
 (A) NAME/KEY: mat\_peptide  
 (B) LOCATION:979..1299  
 (D) OTHER INFORMATION:/product= "VL3418"

(ix) FEATURE:  
 (A) NAME/KEY: misc\_RNA  
 (B) LOCATION:1300..1344  
 (D) OTHER INFORMATION:/product= "linker V  
 (Gly4Ser)2Gly4Val"

(ix) FEATURE:  
 (A) NAME/KEY: mat\_peptide  
 (B) LOCATION:1345..1686  
 (D) OTHER INFORMATION:/product= "VL4715"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AAGCTTGCAT GGAAATTCTA TTTCAAGGAG ACAGTCATA ATG AAA TAC CTA TTG	54
Met Lys Tyr Leu Leu -22 -20	
CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA CCA GCG ATG	102
Pro Thr Ala Ala Ala Gly Leu Leu Leu Ala Ala Gln Pro Ala Met -15 -10 -5	
GCC CAG GTG CAG CTG CAG GAG TCA GGG GGA GAC TTA GTG AAG CCT GGA	150
Ala Gln Val Gln Leu Gln Glu Ser Gly Gly Asp Leu Val Lys Pro Gly 1 5 10 15	
GGG TCC CTG ACA CTC TCC TGT GCA ACC TCT GGA TTC ACT TTC AGT AGT	198
Gly Ser Leu Thr Leu Ser Cys Ala Thr Ser Gly Phe Thr Phe Ser Ser 20 25 30	
TAT GCC TTT TCT TGG GTC CGC CAG ACC TCA GAC AAG AGT CTG GAG TGG	246
Tyr Ala Phe Ser Trp Val Arg Gln Thr Ser Asp Lys Ser Leu Glu Trp 35 40 45	
GTC GCA ACC ATC AGT AGT ACT GAT ACT TAT ACC TAT TAT TCA GAC AAT	294
Val Ala Thr Ile Ser Ser Thr Asp Thr Tyr Thr Tyr Tyr Ser Asp Asn 50 55 60	
GTG AAG GGG CGC TTC ACC ATC TCC AGA GAC AAT GGC AAG AAC ACC CTG	342
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Gly Lys Asn Thr Leu 65 70 75	
TAC CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC ACA GCC GTG TAT TAC	390
Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Val Tyr Tyr 80 85 90 95	
TGT GCA AGA CAT GGG TAC TAT GGT AAA GGC TAT TTT GAC TAC TGG GGC	438
Cys Ala Arg His Gly Tyr Tyr Gly Lys Gly Tyr Phe Asp Tyr Trp Gly 100 105 110	
CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA	486
Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly 115 120 125	
GGT GGC TCT GGC GGT GGC GGA TCG GCC GGT TCG GCC CAG GTC CAG CTG	534
Gly Gly Ser Gly Gly Gly Gly Ser Ala Gly Ser Ala Gln Val Gln Leu 130 135 140	



55

CAA Gln 145	CAG Gln 145	TCA Ser 145	GGA Gly 145	CCT Pro 145	GAG Glu 150	CTG Leu 150	GTA Val 150	AAG Lys 150	CCT Pro 150	GGG Gly 155	GCT Ala 155	TCA Ser 155	GTG Val 155	AAG Lys 155	ATG Met 155	582
TCC Ser 160	TGC Cys 160	AAG Lys 160	GCT Ala 160	TCT Ser 160	GGA Gly 165	TAC Tyr 165	ACA Thr 165	TTC Phe 165	ACT Thr 170	AGC Ser 170	TAT Tyr 170	GTT Val 170	ATG Met 175	CAC His 175	TGG Trp 175	630
GTG Val 180	AAA Lys 180	CAG Gln 180	AAG Lys 180	CCT Pro 180	GGG Gly 180	CAG Gln 185	GGC Gly 185	CTT Leu 185	GAG Glu 185	TGG Trp 185	ATT Ile 190	GGA Gly 190	TAT Tyr 190	ATT Ile 190	TAT Tyr 190	678
CCT Pro 195	TAC Tyr 195	AAT Asn 195	GAT Asp 195	GGT Gly 195	ACT Thr 195	AAG Lys 200	TAC Tyr 200	AAT Asn 200	GAG Glu 200	AAG Lys 205	TTC Phe 205	AAA Lys 205	GGC Gly 205	AAG Lys 205	GCC Ala 205	726
ACA Thr 210	CTG Leu 210	ACT Thr 210	TCA Ser 210	GAC Asp 210	AAA Lys 215	TCC Ser 215	TCC Ser 215	AGC Ser 215	ACA Thr 215	GCC Ala 220	TAC Tyr 220	ATG Met 220	GAG Glu 220	CTC Leu 220	AGC Ser 220	774
AGC Ser 225	CTG Leu 225	ACC Thr 225	TCT Ser 225	GAG Glu 230	GAC Asp 230	TCT Ser 230	GCG Ala 230	GTC Val 230	TAT Tyr 230	TAC Tyr 235	TGT Cys 235	TCA Ser 235	AGA Arg 235	CGC Arg 235	TTT Phe 235	822
GAC Asp 240	TAC Tyr 240	TGG Trp 240	GGC Gly 245	CAA Gln 245	GGG Gly 245	ACC Thr 245	ACC Thr 245	GTC Val 250	ACC Thr 250	GTC Val 250	TCC Ser 250	TCA Ser 250	TAA *			864
TAAGCTAGCG GAGCTGCATG CAAATTCTAT TTCAAGGAGA CAGTCATA ATG AAA TAC Met Lys Tyr -22 -20																921
CTA Leu -15	TTG Leu -15	CCT Pro -15	ACG Thr -15	GCA Ala -15	GCC Ala -15	GCT Ala -15	GGA Gly -10	TTG Leu -10	TTA Leu -10	CTC Leu -5	GCT Ala -5	GCC Ala -5	CAA Gln -5	CCA Pro -5		969
GCG Ala 1	ATG Met 1	GCC Ala 1	GAC Asp 1	ATC Ile 1	GAG Glu 5	CTC Leu 5	ACC Thr 5	CAG Gln 5	TCT Ser 10	CCA Pro 10	TCT Ser 10	TCC Ser 10	ATG Met 10	TAT Tyr 10	GCA Ala 10	1017
TCT Ser 15	CTA Leu 15	GGA Gly 15	GAG Glu 15	AGA Arg 20	ATC Ile 20	ACT Thr 20	ATC Ile 20	ACT Thr 20	TGC Cys 25	AAG Lys 25	GCG Ala 25	AGT Ser 25	CAG Gln 25	GAC Asp 25	ATT Ile 25	1065
AAT Asn 30	ACC Thr 30	TAT Tyr 30	TTA Leu 35	ACC Thr 35	TGG Trp 35	TTC Phe 35	CAG Gln 40	CAG Gln 40	AAA Lys 40	CCA Pro 40	GGG Gly 40	AAA Lys 40	TCT Ser 45	CCC Pro 45	AAG Lys 45	1113
ACC Thr 50	CTG Leu 50	ATC Ile 50	TAT Tyr 50	CGT Arg 50	GCA Ala 50	AAC Asn 55	AGA Arg 55	TTG Leu 55	CTA Leu 55	GAT Asp 55	GGG Gly 60	GTC Val 60	CCA Pro 60	TCA Ser 60	AGG Arg 60	1161
TTC Phe 65	AGT Ser 65	GGC Gly 65	AGT Ser 65	GGA Gly 65	TCT Ser 70	GGG Gly 70	CAA Gln 70	GAT Asp 70	TAT Tyr 70	TCT Ser 75	CTC Leu 75	ACC Thr 75	ATC Ile 75	AGC Ser 75	AGC Ser 75	1209
CTG Leu 80	GAC Asp 80	TAT Tyr 80	GAA Glu 80	GAT Asp 85	ATG Met 85	GGA Gly 85	ATT Ile 85	TAT Tyr 85	TAT Tyr 85	TGT Cys 90	CTA Leu 90	CAA Gln 90	TAT Tyr 90	GAT Asp 90	GAG Glu 90	1257
TTG Leu 95	TAC Tyr 95	ACG Thr 95	TTC Phe 95	GGA Gly 100	GGG Gly 100	GGG Gly 100	ACC Thr 100	AAG Lys 100	CTC Leu 105	GAG Glu 105	ATC Ile 105	AAA Lys 105	CGG Arg 105	GGT Gly 105	GGA Gly 105	1305
GGC Gly 110	GGT Gly 110	TCA Ser 110	GGC Gly 115	GGA Gly 115	GGT Gly 115	GGC Gly 115	TCT Ser 115	GGC Gly 115	GGT Gly 115	GGC Gly 120	GGA Gly 120	GTC Val 120	GAC Asp 120	ATC Ile 120	GAA Glu 125	1353

CTC	ACT	CAG	TCT	CCA	TTC	TCC	CTG	ACT	GTG	ACA	GCA	GGA	GAG	AAG	GTC	1401
Leu	Thr	Gln	Ser	Pro	Phe	Ser	Leu	Thr	Val	Thr	Ala	Gly	Glu	Lys	Val	
				130					135					140		
ACT	ATG	AAT	TGC	AAG	TCC	GGT	CAG	AGT	CTG	TTA	AAC	AGT	GTA	AAT	CAG	1449
Thr	Met	Asn	Cys	Lys	Ser	Gly	Gln	Ser	Leu	Leu	Asn	Ser	Val	Asn	Gln	
			145					150					155			
AGG	AAC	TAC	TTG	ACC	TGG	TAC	CAG	CAG	AAG	CCA	GGG	CAG	CCT	CCT	AAA	1497
Arg	Asn	Tyr	Leu	Thr	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Pro	Pro	Lys	
			160				165					170				
CTG	TTG	ATC	TAC	TGG	GCA	TCC	ACT	AGG	GAA	TCT	GGA	GTC	CCT	GAT	CGC	1545
Leu	Leu	Ile	Tyr	Trp	Ala	Ser	Thr	Arg	Glu	Ser	Gly	Val	Pro	Asp	Arg	
	175					180					185					
TTC	ACA	GCC	AGT	GGA	TCT	GGA	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	AGT	1593
Phe	Thr	Ala	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	
190					195				200					205		
GTG	CAG	GCT	GAA	GAC	CTG	GCA	GTT	TAT	TAC	TGT	CAG	AAT	GAT	TAT	ACT	1641
Val	Gln	Ala	Glu	Asp	Leu	Ala	Val	Tyr	Tyr	Cys	Gln	Asn	Asp	Tyr	Thr	
				210					215					220		
TAT	CCG	TTC	ACG	TTC	GGA	GGG	GGG	ACC	AAG	CTC	GAA	ATC	AAA	CGG	TAA	1689
Tyr	Pro	Phe	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	*	
			225					230					235			
TAAGCGGCCG	CGAATTC															1706

## (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Met	Lys	Tyr	Leu	Leu	Pro	Thr	Ala	Ala	Ala	Gly	Leu	Leu	Leu	Ala	
-22		-20					-15					-10			
Ala	Gln	Pro	Ala	Met	Ala	Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Asp
-5					1			5							10
Leu	Val	Lys	Pro	Gly	Gly	Ser	Leu	Thr	Leu	Ser	Cys	Ala	Thr	Ser	Gly
				15				20						25	
Phe	Thr	Phe	Ser	Ser	Tyr	Ala	Phe	Ser	Trp	Val	Arg	Gln	Thr	Ser	Asp
			30					35					40		
Lys	Ser	Leu	Glu	Trp	Val	Ala	Thr	Ile	Ser	Ser	Thr	Asp	Thr	Tyr	Thr
		45				50					55				
Tyr	Tyr	Ser	Asp	Asn	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn
	60					65					70				
Gly	Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Ser	Ser	Leu	Lys	Ser	Glu	Asp
	75					80				85				90	
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	His	Gly	Tyr	Tyr	Gly	Lys	Gly	Tyr
				95					100					105	

(2) INFORMATION FOR SEQ ID NO: 13:

(A) LENGTH: 259 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

BNSDOCID: &lt;WO\_9714719A1\_1&gt;

58

Asp Ile Glu Leu Thr Gln Ser Pro Phe Ser Leu Thr Val Thr Ala Gly  
           125                                  130                                  135  
 Glu Lys Val Thr Met Asn Cys Lys Ser Gly Gln Ser Leu Leu Asn Ser  
           140                                  145                                  150  
 Val Asn Gln Arg Asn Tyr Leu Thr Trp Tyr Gln Gln Lys Pro Gly Gln  
           155                                  160                                  165                                  170  
 Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser Gly Val  
                                   175                                  180                                  185  
 Pro Asp Arg Phe Thr Ala Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr  
                                   190                                  195                                  200  
 Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Asn  
                                   205                                  210                                  215  
 Asp Tyr Thr Tyr Pro Phe Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile  
           220                                  225                                  230  
 Lys Arg \*  
 235

## (2) INFORMATION FOR SEQ ID NO: 14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: primer DBL.1

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CACCATCTCC AGAGACAATG GCAAG

25

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: primer DBL.2

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GAGCGCGAGC TCGGCCGAAC CGGCCGATCC GCCACCGCCA GAGCC

45

## (2) INFORMATION FOR SEQ ID NO: 16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs

59

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.3

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CAGGATCCGG CCGGTTCCGC CCAGGTCCAG CTGCAACAGT CAGGA

45

- (2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 53 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.4

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CTACATGAAT TCGCTAGCTT ATTATGAGGA GACGGTGACG GTGGTCCCTT GGC

53

- (2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.5

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TAATAAGCTA GCGGAGCTGC ATGCAAATTC TATTTTC

36

- (2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ACCAAGCTCG AGATCAAACG GGG

23

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer DBL.7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AATGTCGAAT TCGTCGACTC CGCCACCGCC AGAGCC

36

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer DBL.8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ATTGGAGTCG ACATCGAACT CACTCAGTCT CCATTCTCC

39

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer DBL.9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TGAAGTGAAT TCGCGGCCGC TTATTACCGT TTGATTTCGA GCTTGGTCCC

50

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid

61

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.10

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CGAATTCGGT CACCGTCTCC TCACAGGTCC AGTTGCAACA G

41

## (2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.11

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGAATTCTCG AGATCAAACG GGACATCGAA CTCACTCAGT CTCC

44

## (2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 41 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer DBL.12

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CGAATTCGGT CACCGTCTCC TCACAGGTGC AGTTCAGGA G

41

## (2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: primer PCR.51

62

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

AGGTSMMAMCT GCAGSAGTCW GG

22

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer PCR.89

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CC

32

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer PCR.90

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GACATTGAGC TCACCCAGTC TCCA

24

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic DNA"

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer PCR.116

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GTTAGATCTC GAGCTTGGTC CC

22

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid



(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Thr Thr Val Thr Val Ser Ser Gln Val Gln Leu Gln Gln  
1                      5                      10

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Lys Leu Glu Ile Lys Arg Asp Ile Glu Leu Thr Gln  
1                      5                      10

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page 14

BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To Dr. P van der Logt  
Unilever Research  
Colworth Laboratory  
Biosciences Division  
Colworth House, Sharnbrook  
Bedford MK44 1LQ

NAME AND ADDRESS  
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  <b>Escherichia coli</b> <b>ScFy 4715.myc</b>	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  <b>NCTC 12916</b>
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 14th October 1995 (date of original deposit) <sup>1</sup>	
<b>IV. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: National Collection of Type Cultures  Address: Central Public Health Laboratory 61 Colindale Avenue London NW9 5HT	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 4/12/95 <i>Barry Holmes</i> <b>B Holmes</b> <b>Clinical Scientist</b>

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

EP/A/II/12  
page 24BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

## INTERNATIONAL FORM

Dr P van der Logt  
Bayer Research  
Wellworth Laboratory  
Biosciences Division  
Wellworth House, Sharnbrook  
Bedford MK44 1LQ

VIABILITY STATEMENT  
issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

NAME AND ADDRESS OF THE PARTY  
WHICH THE VIABILITY STATEMENT  
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
<p>Dr P van der Logt</p> <p>As above</p>	<p>Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12916</p> <p>Date of the deposit or of the transfer: 14th October 1995</p>
<b>1. VIABILITY STATEMENT</b>	
<p>1. Viability of the microorganism identified under II above was tested</p> <p>2. On that date, the said microorganism was</p> <p><input type="checkbox"/> viable</p> <p><input type="checkbox"/> no longer viable</p>	

Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

Mark with a cross the applicable box.

on 21/9 (first page)

SP/A/II/12  
page 25IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

Nutrient Agar without additions (a)  
Agar (17g Bacto-Tryptone, 10g Bacto-Yeast Extract, 5g NaCl, per  
litre) with 100ug/ml ampicillin and 1% glucose (b)  
Aerobic incubation, 37°C, 24 hrs  
(a)  $8 \times 10^5$  cfu/ml  
(b)  $6 \times 10^5$  cfu/ml

## INTERNATIONAL DEPOSITARY AUTHORITY

Name:

National Collection of Type Cultures  
Central Public Health Laboratory  
61 Colindale Avenue, London NW9 5HT  
Telephone: 0181-300 4400  
Telex: 6953942 (DEPHEM G)  
Fax: 0181-300 7874

Address:

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Date: 4/12/95

*Barry Holmes*  
B Holmes  
Clinical Scientist

Fill in if the information has been requested and if the results of the test were negative.

BP/A/II/12  
page 14BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

## INTERNATIONAL FORM

to Dr. P van der Logt  
Unilever Research  
Colworth Laboratory  
Biosciences Division  
Colworth House, Sharnbrook  
Bedford MK44 1LQ

NAME AND ADDRESS  
OF DEPOSITORRECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:  Escherichia coli Fv 3418	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  NCTC 12915
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:  <input type="checkbox"/> a scientific description  <input checked="" type="checkbox"/> a proposed taxonomic designation  (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 14th October 1995 (date of original deposit) <sup>1</sup>	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: National Collection of Type Cultures  Address: Central Public Health Laboratory 61 Colindale Avenue London NW9 5HT	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  date: 4/12/95      Barry Holmes B Holmes Clinical Scientist

<sup>1</sup> Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

BP/A/II/12  
page 24

VIENNA TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Dr P van der Logt  
Biliver Research  
Bilworth Laboratory  
Biosciences Division  
Bilworth House, Sharnbrook  
Bedford MK44 1LQ

VIABILITY STATEMENT  
issued pursuant to Rule 10.2 by the  
INTERNATIONAL DEPOSITARY AUTHORITY  
identified on the following page

NAME AND ADDRESS OF THE PARTY  
WHICH THE VIABILITY STATEMENT  
IS ISSUED

I. DEPOSIT	II. IDENTIFICATION OF THE MICROORGANISM
Name: Dr P van der Logt  Address: As above	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12915  Date of the deposit or of the transfer: 14th October 1995

A. VIABILITY STATEMENT

1. Viability of the microorganism identified under II above was tested  
20th November 1995
2. On that date, the said microorganism was
- ☒ viable
- ☐ no longer viable

Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

Please tick a cross the applicable box.

See also (other pages)

PCT/EP96/03605  
page 25III. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED<sup>4</sup>

Nutrient Agar without additions (a)  
Agar (17g Bacto-Tryptone, 10g Bacto-Yeast Extract, 5g NaCl, per  
litre) with 100ug/ml ampicillin and 1% glucose (b)  
Aerobic incubation, 37°C, 24 hrs.

(a)  $2 \times 10^6$  cfu/ml(b)  $1 \times 10^6$  cfu/ml

## INTERNATIONAL DEPOSITARY AUTHORITY

Address: National Collection of Type Cultures  
Central Public Health Laboratory  
61 Colindale Avenue, London NW9 5HT  
Telephone: 0181-200 4400  
Telex: 8933942 (DEFEND G)  
Fax: 0181-200 7574

Signature(s) of person(s) having the power  
to represent the International Depositary  
Authority or of authorized official(s):

Date: 4/12/95

*Barry Holmes*  
B Holmes  
Clinical Scientist

Fill in if the information has been requested and if the results of the test were negative.

## C L A I M S

1. A **bispecific** or **bivalent** antibody fragment analogue, which comprises a binding complex containing two polypeptide chains, whereby one polypeptide chain comprises two times a variable domain of a heavy chain ( $V_H$ ) **in series** and the other polypeptide chain comprises two times a variable domain of a light chain ( $V_L$ ) **in series**, and the binding complex contains two pairs of variable domains ( $V_H$ -A// $V_L$ -A and  $V_H$ -B// $V_L$ -B).
2. An antibody fragment analogue according to claim 1, in which one polypeptide chain comprises a first  $V_H$  connected to a second  $V_H$  and the other polypeptide chain comprises a first  $V_L$  connected to a second  $V_L$ .
3. An antibody fragment analogue according to claim 2, in which the two  $V_H$ 's are directly connected to each other without an intermediate peptide linker.
4. An antibody fragment analogue according to claim 2, in which the two  $V_L$ 's are directly connected to each other without an intermediate peptide linker.
5. An antibody fragment analogue according to claim 3 or claim 4, in which one polypeptide chain comprises a first  $V_H$  directly connected to a second  $V_H$ , and the other polypeptide chain comprises a first  $V_L$  directly connected to a second  $V_L$ .
6. An antibody fragment analogue according to claim 2, in which the two  $V_H$ 's are connected to each other by a peptide linker and also the two  $V_L$ 's are connected to each other by a peptide linker, each peptide linker comprising at least one amino acid residue.



7. An antibody fragment analogue according to claim 6, in which one polypeptide chain comprises a first  $V_H$  ( $V_H$ -A) followed by a second  $V_H$  ( $V_H$ -B) and the other polypeptide chain comprises a first  $V_L$  ( $V_L$ -A) followed by a second  $V_L$  ( $V_L$ -B), and in which the two  $V_H$ 's are connected to each other by a peptide linker ( $Li_H$ ), thus [ $V_H$ -A \*  $Li_H$  \*  $V_H$ -B], and also the two  $V_L$ 's are connected to each other by a peptide linker ( $Li_L$ ), thus [ $V_L$ -A \*  $Li_L$  \*  $V_L$ -B], each peptide linker comprising at least 10 amino acid residues.

8. An antibody fragment analogue according to claim 2, in which one polypeptide chain comprises a first  $V_H$  ( $V_H$ -A) followed by a second  $V_H$  ( $V_H$ -B) with or without a connecting peptide linker ( $Li_H$ ), thus [ $V_H$ -A \* ( $Li_H$ ) \*  $V_H$ -B], and the other polypeptide chain comprises a first  $V_L$  ( $V_L$ -A) preceded by a second  $V_L$  ( $V_L$ -B) with or without a connecting peptide linker ( $Li_L$ ), thus [ $V_L$ -B \* ( $Li_L$ ) \*  $V_L$ -A].

9. An antibody fragment analogue according to claim 1, in which the two variable domains are different resulting in a **bispecific** antibody fragment analogue.

10. An antibody fragment analogue according to claim 1, in which the specificities A and B are the same resulting in a **bivalent** antibody fragment analogue.

11. Use of an antibody fragment analogue according to claim 1, in immunoassays including diagnostic techniques, in agglutination assays, in a purification method, for compositions suitable for therapy, or in other methods in which immunoglobulins or fragments thereof can be used.

12. A process for producing an antibody fragment analogue according to any one of claims 1-10, which comprises

(1) transforming a host by incorporating into that host a DNA encoding the two  $V_H$ 's in series with or without a

connecting peptide linker and a DNA encoding the two  $V_L$ 's in series with or without a connecting peptide linker,

(2) culturing such transformed host under conditions whereby the connected  $V_H$ 's and the connected  $V_L$ 's are formed, and

(3) allowing the two connected  $V_H$ 's and the two connected  $V_L$ 's to combine to each other under formation of a double head antibody fragment analogue, and

(4) optionally collecting the double head antibody fragment analogue.

13. A process for producing an antibody fragment analogue according to any one of claims 1-10, which comprises

(1) transforming a first host by incorporating into that first host a DNA encoding the two  $V_H$ 's in series with or without a connecting peptide linker,

(2) transforming a second host by incorporating into that second host a DNA encoding the two  $V_L$ 's in series with or without a connecting peptide linker,

(3) culturing the first and second transformed host under conditions whereby the connected  $V_H$ 's and the connected  $V_L$ 's, respectively, are formed,

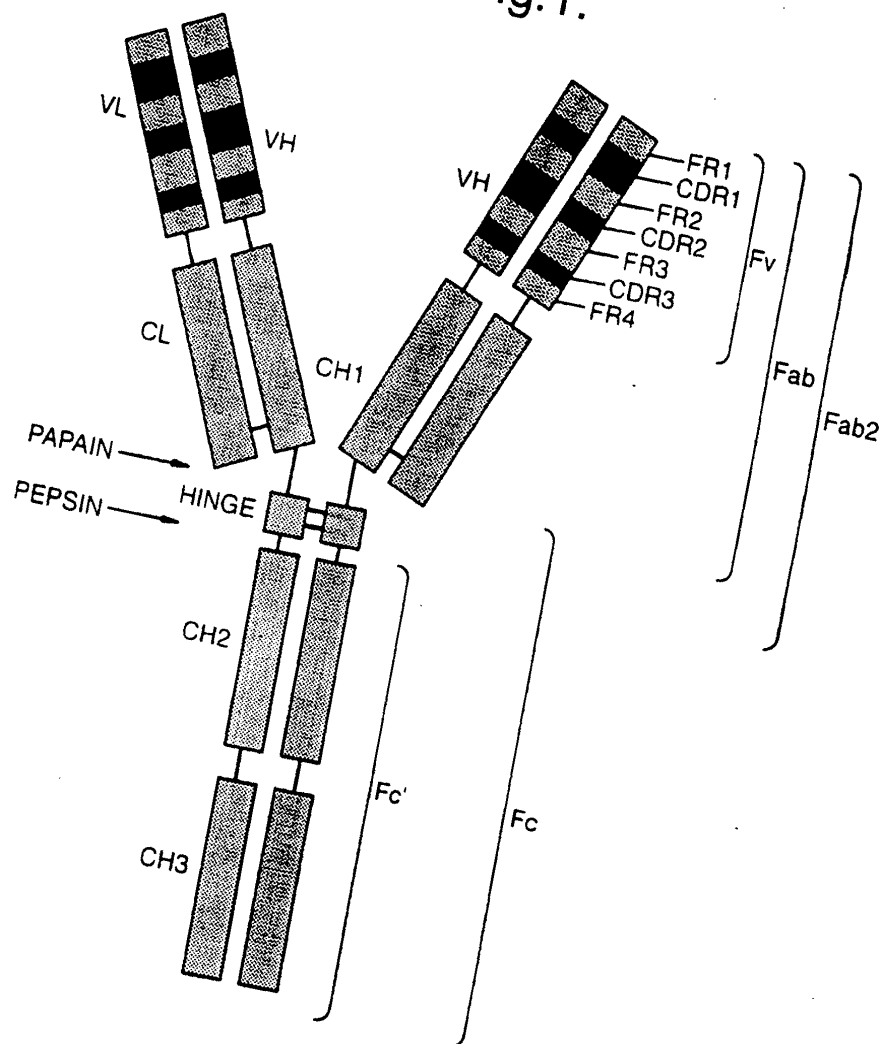
(4) optionally collecting separately the two connected  $V_H$ 's and the two connected  $V_L$ 's, and

(5) combining the connected  $V_H$ 's and the connected  $V_L$ 's under conditions that they can form a double head antibody fragment analogue.

14. A process according to claim 12 or 13, in which the host is selected from the group consisting of prokaryotic micro-organisms comprising Gram-negative bacteria (e.g. *E. coli*) and Gram-positive bacteria (e.g. *B. subtilis* or lactic acid bacteria), lower eukaryotic microorganisms comprising yeasts (e.g. belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, and *Hansenula*) and moulds (e.g. belonging to the genera *Aspergillus*,

*Neurospora* or *Trichoderma*), and higher eukaryotic organisms (e.g. plants) or cell cultures thereof (e.g. hybridoma's).

Fig. 1.



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Fig.2A.



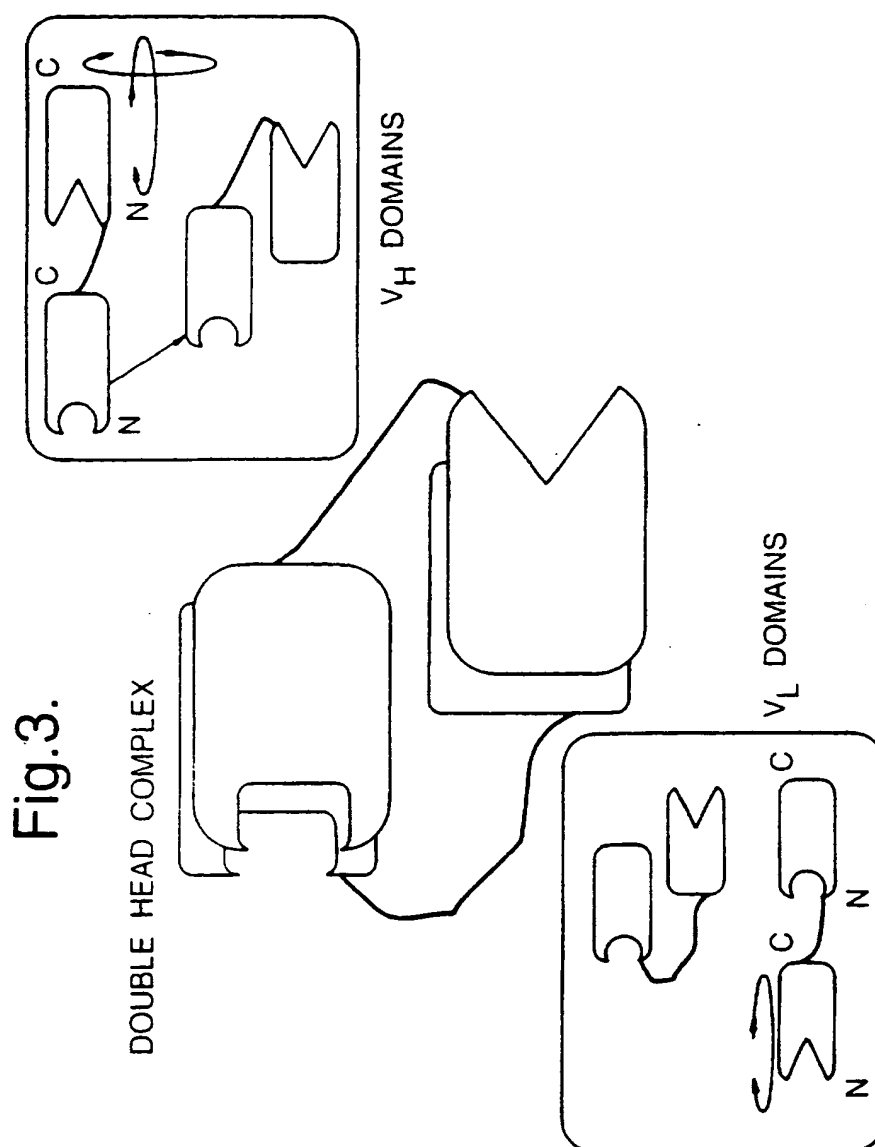
Fig.2B.



Fig.2C.



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SUBSTITUTE SHEET (RULE 26)

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Fig.4.

D I E L T Q S P A S L S A S V G E  
GAATTCGGCCGACATCGAGCTCACCAGTCTCCAGCCTCCCTTTCTGCGTCTGTGGGAGA  
 EcoRI SacI 60

T V T I T C R A S G N I H N Y L A W Y Q  
 AACTGTCACCATCACATGTCGAGCAAGTGGGAATATTCACAATTATTTAGCATGGTATCA  
 120

Q K Q G K S P Q L L V Y Y T T T L A D G  
 GCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATTATACAACAACCTTAGCAGATGG  
 180

V P S R F S G S G S G T Q Y S L K I N S  
 TGTGCCATCAAGGTTTCAGTGGCAGTGGATCAGGAACACAATATTCTCTCAAGATCAACAG  
 240

L Q P E D F G S Y Y C Q H F W S T P R T  
 CCTGCAACCTGAAGATTTTGGGAGTTATTACTGTCAACATTTTGGAGTACTCCTCGGAC  
 300

F G G G T K L E I K R G G G G S G G G G  
 GTTCGGTGGAGGGACCAAGCTCGAGATCAAACGGGGTGGAGGCGGTTTCAGGCGGAGGTGG  
 XhoI 360

S G G G G S Q V Q L Q E S G P G L V A P  
 CTCTGGCGGTGGCGGATCGCAGGTGCAGCTGCAGGAGTCAGGACCTGGCCTGGTGGCGCC  
 PstI 420

S Q S L S I T C T V S G F S L T G Y G V  
 CTCACAGAGCCTGTCCATCACATGCACCGTCTCAGGGTTCTCATTAAACGGCTATGGTGT  
 480

N W V R Q P P G K G L E W L G M I W G D  
 AAAGTGGGTTCCGCCAGCCTCCAGGAAAGGGTCTGGAGTGGCTGGGAATGATTTGGGGTGA  
 540

G N T D Y N S A L K S R L S I S K D N S  
 TGGAAACACAGACTATAATTAGCTCTCAAATCCAGACTGAGCATCAGCAAGGACAACCTC  
 600

K S Q V F L K M N S L H T D D T A R Y Y  
 CAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCACACTGATGACACAGCCAGGTACTA  
 660

C A R E R D Y R L D Y W G Q G T T V T V  
 CTGTGCCAGAGAGAGAGATTATAGGCTTGACTACTGGGGCCAAGGGACCACGGTCACCGT  
 720

S S \*  
 CTCCTCATGATAAGCTT  
 HindIII 737

DIELTQSPAS---GGGTKLEIKR = VLLys  
 GGGSGGGSGGGGS = Linker  
 QVQLQESGPG---GQGTVTVSS = VHLys

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Fig.5.

M K Y L L P T A  
 pelB AAGCTTGCAAATTCTATTTCAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAG  
 LEADER A A G L L L L A A Q P A M A Q V Q L Q Q  
 CCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCAGGTGCAGCTGCAGCAGT  
 S G P E L V K P G A S V R M S C K A S G  
 CAGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCTTCTGGAT  
 Y T F T S Y V M H W V K Q K P G Q G L R  
 ACACATTCAGTAGCTATGTTATGCACTGGGTGAAACAGAAGCCTGGGCAGGGCCTTGAGT  
 VH3418 W I G Y I Y P Y N D G T K Y N E K F K G  
 GGATTGGATATATTTATCCTTACAATGATGGTACTAAGTACAATGAGAAGTTCAAAGGCA  
 K A T L T S D K S S S T A Y M E L S S L  
 AGGCCACACTGACTTCAGACAAATCCTCCAGCACAGCCTACATGGAGCTCAGCAGCCTGA  
 T S E D S A V Y Y C S R R F D Y W G Q G  
 CCTCTGAGGACTCTGCGGTCTATTACTGTTCAAGACGCTTTCAGTACTGGGGCCAAGGGA  
 T T V T V S S  
 CCACGGTCACCGTCTCCTCATAATAAGAGCTATGGGAGCTTGCATGCAAATTCATTTCAT  
 M K Y L L P T A A A G L L L L  
 pelB AGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCG  
 LEADER A A Q P A M A D I E L T Q S P S S M Y A  
 CTGCCCAACCAGCGATGGCCGACATCGAGCTCAGCCAGTCTCCATCTTCCATGTATGCAT  
 S L G E R I T I T C K A S Q D I N T Y L  
 CTCTAGGAGAGAGAATCACTATCACTTGCAAGGCGAGTCAGGACATTAATACCTATTAA  
 T W F Q Q K P G K S P K T L I Y R A N R  
 CCTGGTTCCAGCAGAAACCAGGGAAATCTCCCAAGACCCTGATCTATCGTGCAAACAGAT  
 VL3418 L L D G V P S R F S G S G S G Q D Y S L  
 TGCTAGATGGGGTCCCATCAAGGTTCAAGTGGCAGTGGATCTGGGCAAGATTATTCTCTCA  
 T I S S L D Y E D M G I Y Y C E Q Y D E  
 CCATCAGCAGCCTGGACTATGAAGATATGGGAATTTATTATTGTCTACAATATGATGAGT  
 L Y T F G G G T K L E I K R  
 TGTACACGTTCCGAGGGGGGACCAAGCTCGAGATCAAACGGTAATAATGATCAAACGGT  
 ATAAGGATCCAGCTCGAATTC



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Fig.6.

M K Y L L P T  
 pelB AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG  
 LEADER A A A G L L L L A A Q P A M A Q V Q L Q  
 GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAG  
 E S G G D L V K P G G S L T L S C A T S  
 GAGTCAGGGGGAGACTTAGTGAAGCCTGGAGGGTCCCTGACACTCTCCTGTGCAACCTCT  
 G F T P S S Y A F S W V R Q T S D K S L  
 GATTCACTTTTCAGTAGTTATGCCTTTTCTGGGTCCGCCAGACCTCAGACAAGAGTCTG  
 E W V A T I S S T D T Y T Y Y S D N V K  
 VH4715 GAGTGGGTGCAACCATCAGTAGTACTGATACTTATACCTATTATTTCAGACAATGTGAAG  
 G R F T I S R D N G K N T L Y L Q M S S  
 GGGCGCTTCACCATCTCCAGAGACAATGGCAAGAACCCCTGTACCTGCAATGAGCAGT  
 L K S E D T A V Y Y C A R H G Y Y G K G  
 CTGAAGTCTGAGGACACAGCCGTGTATTACTGTGCAAGACATGGGTACTATGGTAAAGGC  
 Y F D Y W G Q G T T V T V S S  
 TATTTTGACTACTGGGGCCAAGGGACCACGCTCCTCCTCATAATAAGAGCTATGG  
 M K Y L L P T  
 pelB GAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG  
 LEADER A A A G L L L L A A Q P A M A D I E L T  
 GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCGACATCGAGCTCACT  
 Q S P F S L T V T A G E K V T M N C K S  
 CAGTCTCCATTCTCCCTGACTGTGACAGCAGGAGAGAAGGTCACTATGAATTGCAAGTCC  
 G Q S L L N S V N Q R N Y L T W Y Q Q K  
 GGTCAGAGTCTGTAAACAGTGTAATCAGAGGAATACTTGACCTSGTACCAGCAGAAG  
 P G Q P P K L L I Y W A S T R E S G V P  
 VL4715 CCAGGGCAGCCTCCTAAACTGTTGATCTACTGGGCATCCACTAGGGAATCTGGAGTCCCT  
 D R F T A S G S G T D F T L T I S S V Q  
 GATCGCTTCACAGCCAGTGGATCTGGAACAGATTTCACTCTCACCATCAGCAGTGTGCAG  
 A E D L A V Y Y C Q N D Y T Y P F T F G  
 GCTGAAGACCTGGCAGTTTATTACTGTGAGAATGATTATACTTATCCGTTACGTTCCGA  
 Myc-tag G G T K L E I K R E Q K L I S E E D L N  
 GGGGGGACCAAGCTCGAGATCAAACGGGAACAAAACATCTCAGAAGAGGATCTGAAT  
 TAATAAGATCAAACGGTAATAAGGATCCAGCTCGAATTC

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Fig.7.

M K Y L L P T

peIB AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG

LEADER A A A G L L L L A A Q P A M A Q V Q L Q  
 GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAG  
 E S G G D L V K P G G S L T L S C A T S  
 GAGTCAGGGGGAGACTTAGTGAAGCCTGGAGGGTCCCTGACACTCTCCTGTGCAACCTCT  
 G F T F S S Y A F S W V R Q T S D K S L  
 GGATTCACCTTTCAGTAGTTATGCCTTTTCTTGGGTCCGCCAGACCTCAGACAAGAGTCTG

VH4715 E W V A T I S S T D T Y T Y Y S D N V K  
 GAGTGGGTGCGCAACCATCAGTAGTACTGATACTTATACCTATTATTTCAGACAATGTGAAG  
 G R F T I S R D N G K N T L Y L Q M S S  
 GGGCGCTTCACCATCTCCAGAGACAATGGCAAGAACACCCTGTACCTGCAATGAGCAGT  
 L K S E D T A V Y Y C A R H G Y Y G K G  
 CTGAAGTCTGAGGACACAGCCGTGTATTACTGTGCAAGACATGGGTACTATGGTAAAGGC  
 Y F D Y W G Q G T T V T V S S G G G G S  
 LINKER TATTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTGGAGGCGGTTCA  
 G G G G S G G G G S D I E L T Q S P F S  
 GGCGGAGGTGCTCTGGCGGTGGCGGATCGGACATCGAGCTCACTCAGTCTCCATTCTCC  
 L T V T A G E K V T M N C K S G Q S L L  
 CTGACTGTGACAGCAGGAGAGAAGGTCACTATGAATTGCAAGTCCGGTCAGAGTCTGTTA  
 N S V N Q R N Y L T W Y Q Q K P G Q P P  
 AACAGTGTAATCAGAGGAAGTACTTGACCTGGTACCAGCAGAAGCCAGGGCAGCCTCCT

VL4715 K L L I Y W A S T R E S G V P D R F T A  
 AAAGTGTGATCTACTGGGCATCCACTAGGGAATCTGGAGTCCCTGATCGCTTCACAGCC  
 S G S G T D F T L T I S S V Q A E D L A  
 AGTGGATCTGGAACAGATTTCACCTCTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCA  
 V Y Y C Q N D Y T Y P F T F G G G T K L  
 GTTTATTACTGTCAGAATGATTATACTTATCCGTTACGTTCCGAGGGGGGACCAAGCTC

Myc-tag E I K R E Q K L I S E E D L N  
 GAGATCAAACGGGAACAAAACTCATCTCAGAAGAGGATCTGAATTAATAAGATCAAACG  
 GTAATAAGGATCCAGCTCGAATTC

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Fig.8.1(2)

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M K Y L L P T

AAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG

pelB A A A G L L L L A A Q P A M A Q V Q L Q  
LEADER GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCGATGGCCCAGGTGCAGCTGCAG

E S G G D L V K P G G S L T L S C A T S  
GAGTCAGGGGAGACTTAGTGAAGCCTGGAGGGTCCCTGACACTCTCCTGTGCAACCTCT

VH4715 G F T P S S Y A F S W V R D T S D K S L  
GGATTCACCTTCAGTAGTTATGCCTTTTCTTGGGTCCGCCAGACCTCAGACAAGAGTCTG

E W V A T I S S T D T Y T Y Y S D N V K  
GAGTGGGTGCGCAACCATCAGTAGTACTGATACTTATACCTATTATTTCAGACAATGTGAAG

G R F T I S R D N G K N T L Y L Q M S S  
GGGCGCTTCACCATCTCCAGAGACAATGGCAAGAACACCCTGTACCTGCAAATGAGCAGT

L K S E D T A V Y Y C A R H G Y Y G K G  
CTGAAGTCTGAGGACACAGCCGTGTATTACTGTGCAAGACATGGGTACTATGGTAAAGGC

Y F D Y W G Q G T T V T V S S G G G G S  
TATTTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTGGAGGCGGTTCA

LINKER G G G G S G G G G S A G S A Q V Q L Q Q  
GGCGGAGGTGGCTCTGGCGGTGGCGGATCGGCCGGTTCGGCCAGGTCCAGCTGCAACAG

S G P E L V K P G A S V K M S C K A S G  
TCAGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATGTCTGCAAGGCTTCTGGA

Y T F T S Y V M H W V K Q K P G Q G L E  
TACACATTCAGTATGTTATGCACTGGGTGAAACAGAAGCCTGGGCAGGGCCTTGAG

VH3418 W I G Y I Y P Y N D G T R Y N E K F K G  
TGGATTGGATATATTTATCCTTACAATGATGGTACTAAGTACAATGAGAAGTTCAAAGGC

K A T L T S D K S S S T A Y M E L S S L  
AAGGCCACACTGACTTCAGACAAATCCTCCAGCACAGCCTACATGGAGCTCAGCAGCCTG

T S E D S A V Y Y C S R R F D Y W G Q G  
ACCTCTGAGGACTCTGCGGTCTATTACTGTTCAAGACGCTTTGACTACTGGGGCCAAGGG

T T V T V S S  
ACCACCGTCACCGTCTCCTCATAATAAGCTAGCGGAGCTGCATGCAAATTCTATTTCAAG

pelB M K Y L L P T A A A G L L L L A  
LEADER GAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCT

A Q P A M A D I E L T Q S P S S M Y A S  
GCCCAACCAGCGATGGCCGACATCGAGCTCACCCAGTCTCCATCTTCCATGTATGCATCT

VH3418 L G E R I T I T C K A S Q D I N T Y L T  
CTAGGAGAGAGAATCACTATCACTTGCAAGGCGAGTCAGGACATTAATACCTATTTTAACC

W F Q Q K P G K S P K T L I Y R A N R L  
TGGTTCCAGCAGAAACCAGGGAAATCTCCCAAGACCCTGATCTATCGTGCAAACAGATTG

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Fig.8. 2(2)

LINKER

VL4715

L D G V P S R F S G S G S G Q D Y S L T  
CTAGATGGGGTCCCATCAAGGTTTCAGTGGCAGTGGATCTGGGCAAGATTATTCTCTCACC

I S S L D Y E D M G I Y Y C L O Y D E L  
ATCAGCAGCCTGGACTATGAAGATATGGGAATTTATTATTGTCTACAATATGATGAGTTG

Y T F G G G T K L E I K R G G G G S G G  
TACACGTTCCGAGGGGGGACCAAGCTCGAGATCAAACGGGTGGAGGCGGTTCAGGCCGA

G G S G G G G V D I E L T Q S P F S L T  
GGTGGCTCTGGCGGTGGCGGAGTCGACATCGAACTCACTCAGTCTCCATTCTCCCTGACT

V T A G E K V T M N C K S G Q S L L N S  
GTGACAGCAGGAGAGAAGGTCACTATGAATTGCAAGTCCGGTCAGAGTCTGTTAAACAGT

V N Q R N Y L T W Y Q Q K P G Q P P K L  
GTAAATCAGAGGAACACTTGTGACCTGGTACCAGCAGAAGCCAGGGCAGCCTCCTAAACTG

L I Y W A S T R E S G V P D R F T A S G  
TTGATCTACTGGGCATCCACTAGGGAATCTGGAGTCCCTGATCGCTTCACAGCCAGTGGA

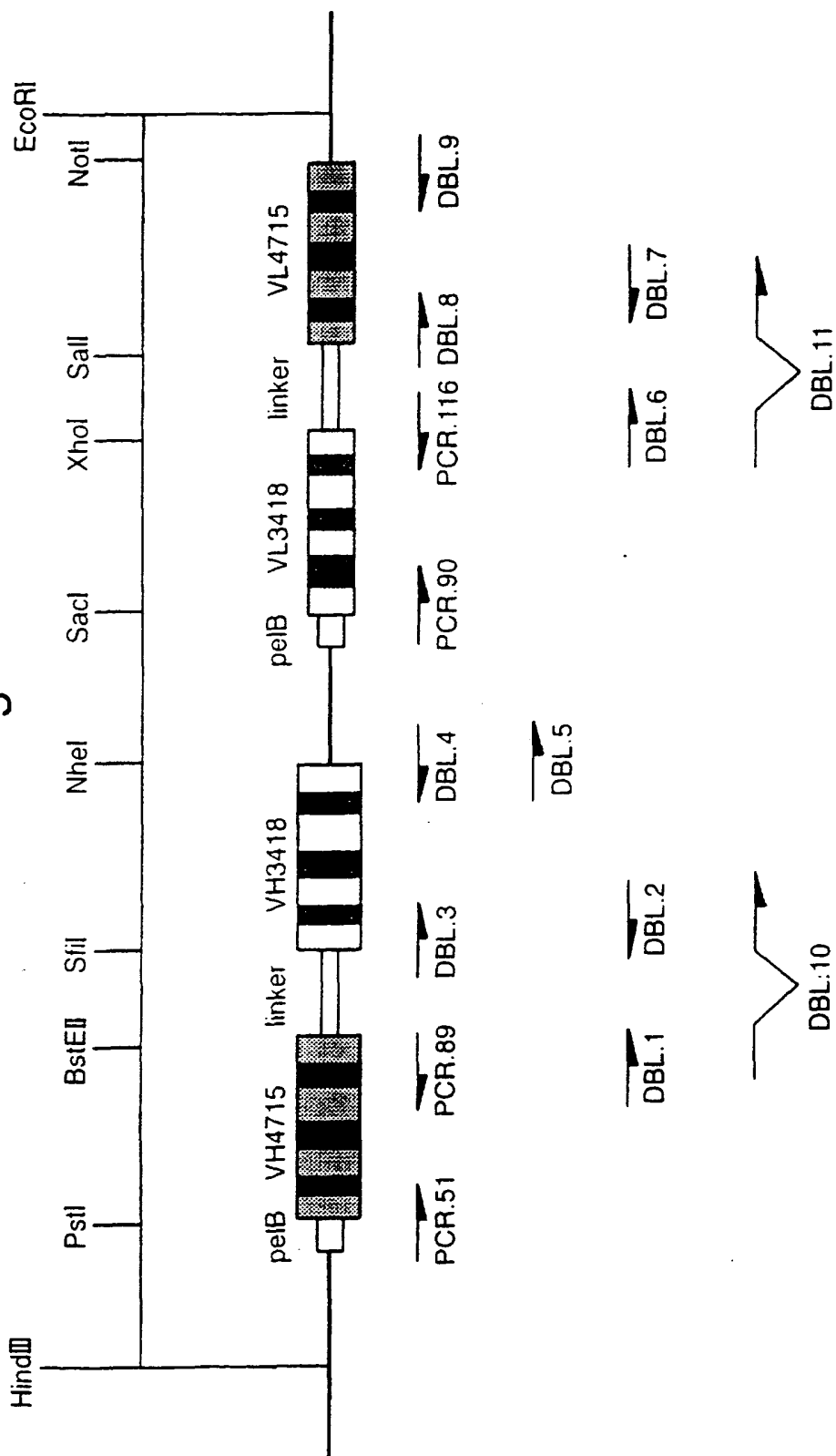
S G T D F T L T I S S V Q A E D L A V Y  
TCTGGAACAGATTTCACTCTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTAT

Y C Q N D Y T Y P F T F G G G T K L E I  
TACTGTCAGAATGATTATACTTATCCGTTACGTTCCGAGGGGGGACCAAGCTCGAAATC

K R  
AAACGGTAATAAGCGGCCGGAATTC

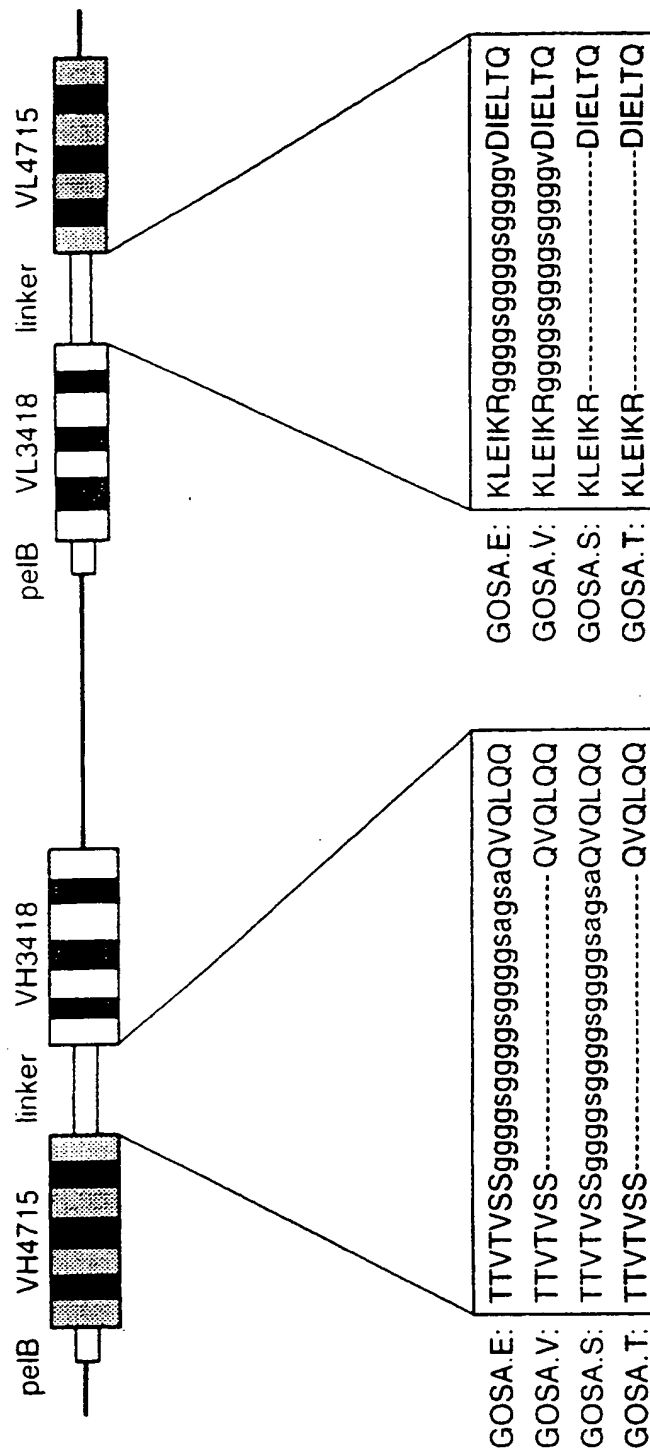
10/45

Fig.9.



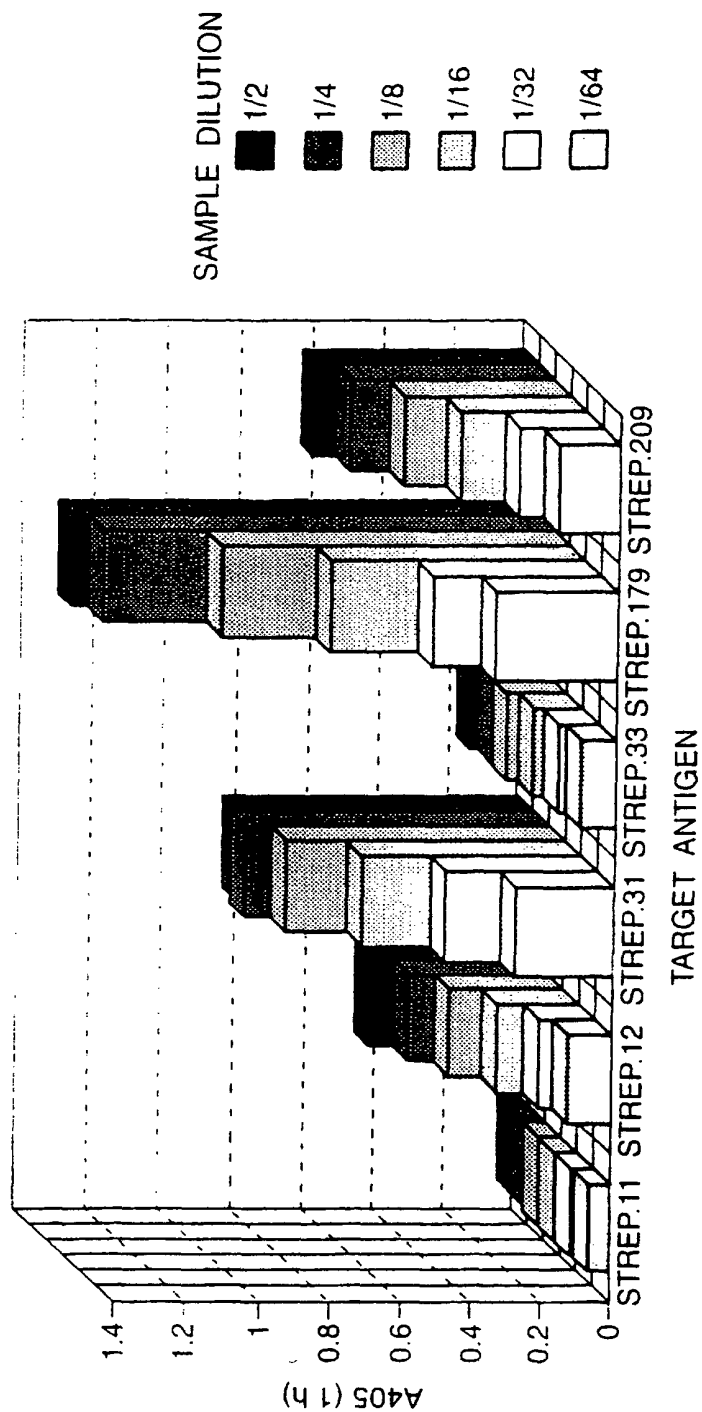
11/45

Fig.10.



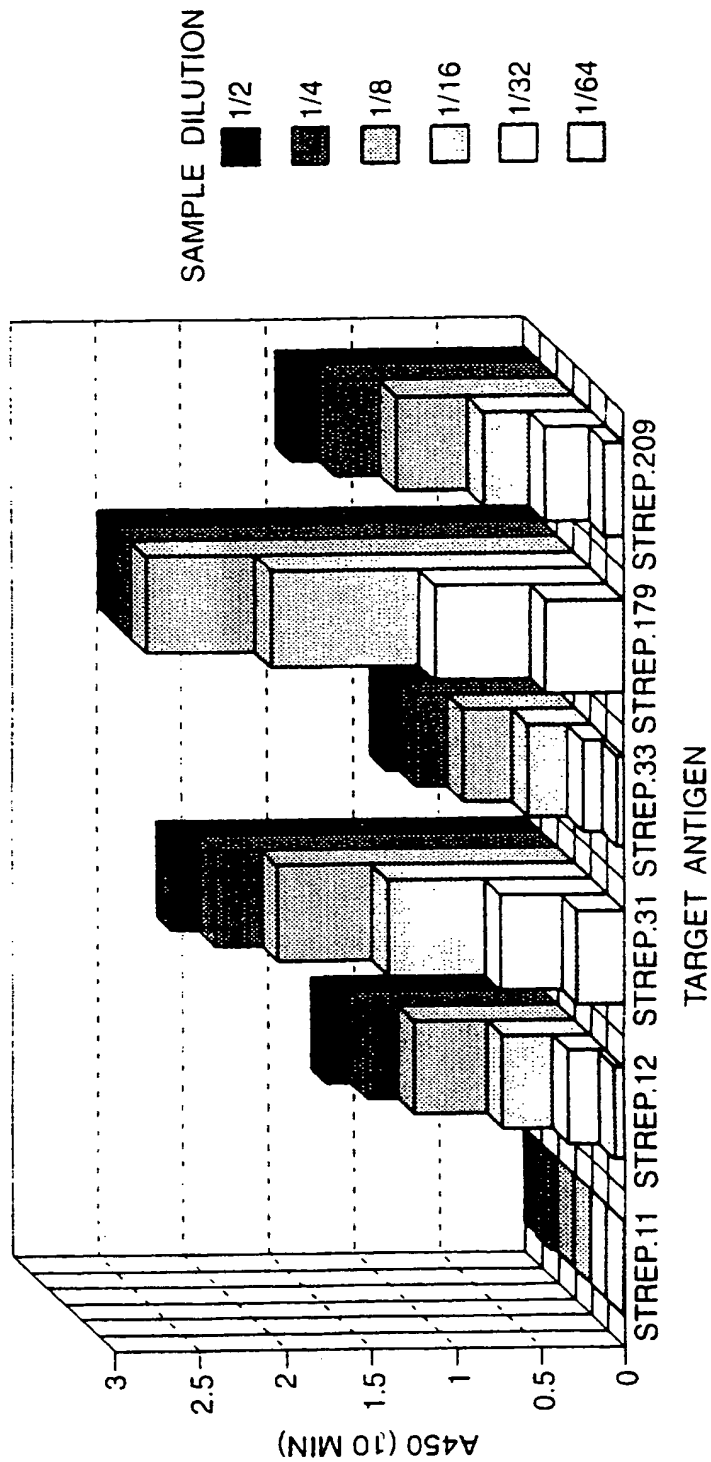
12/45

Fig.11.



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Fig.12.

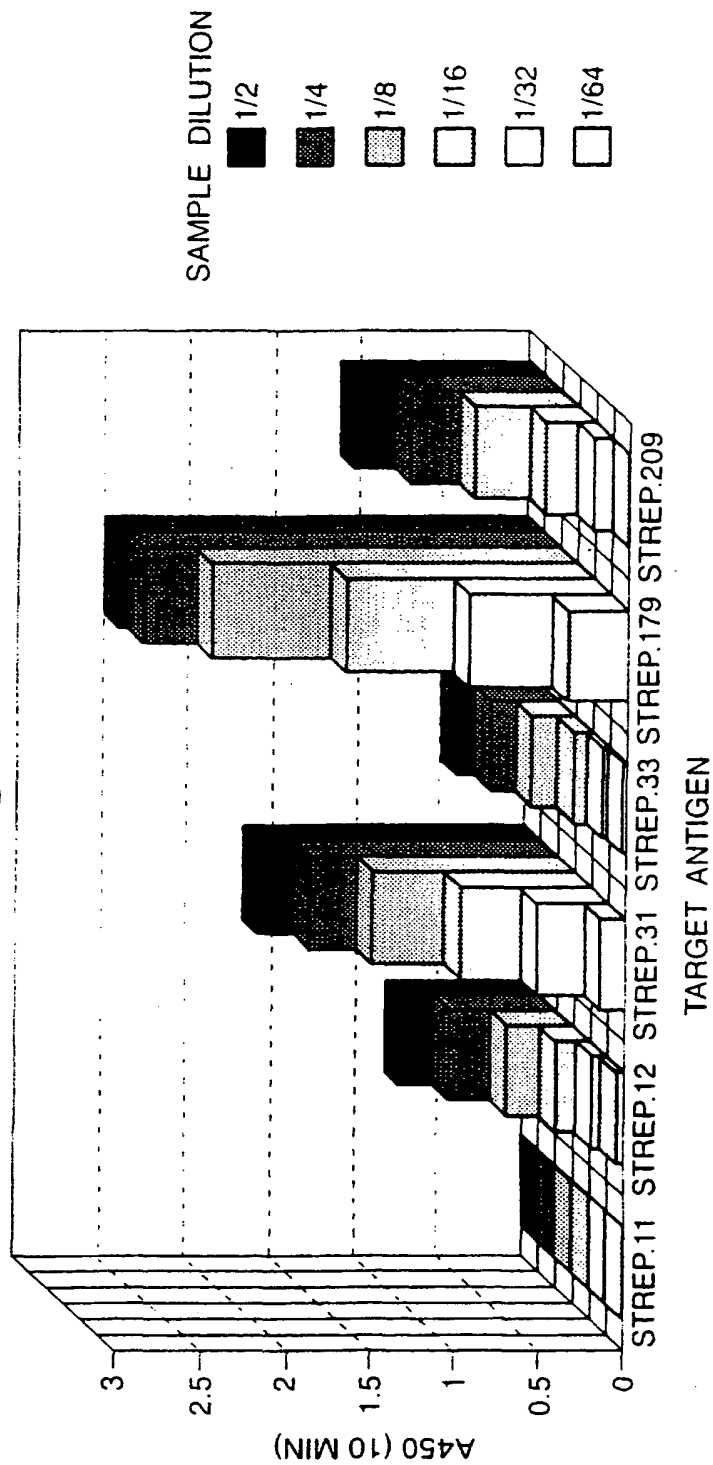


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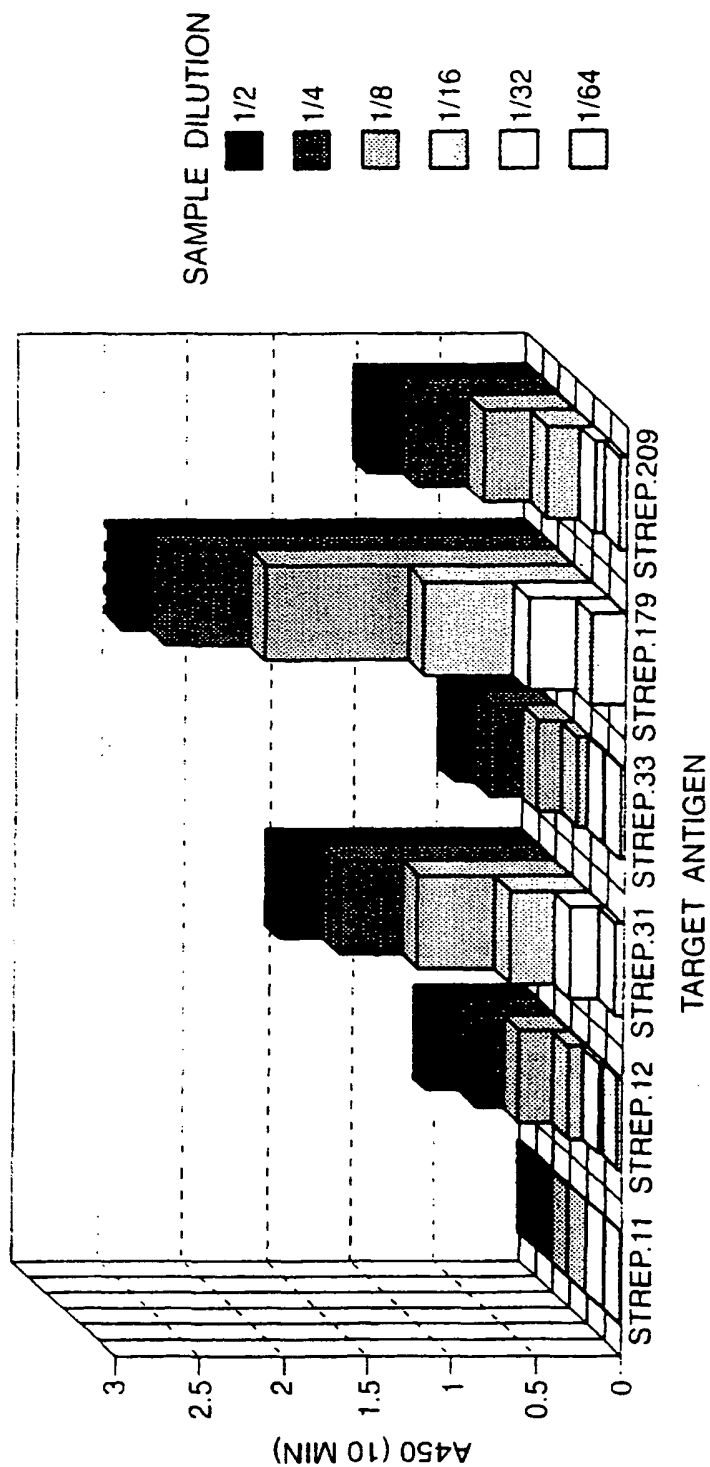
144/45

Fig.13.



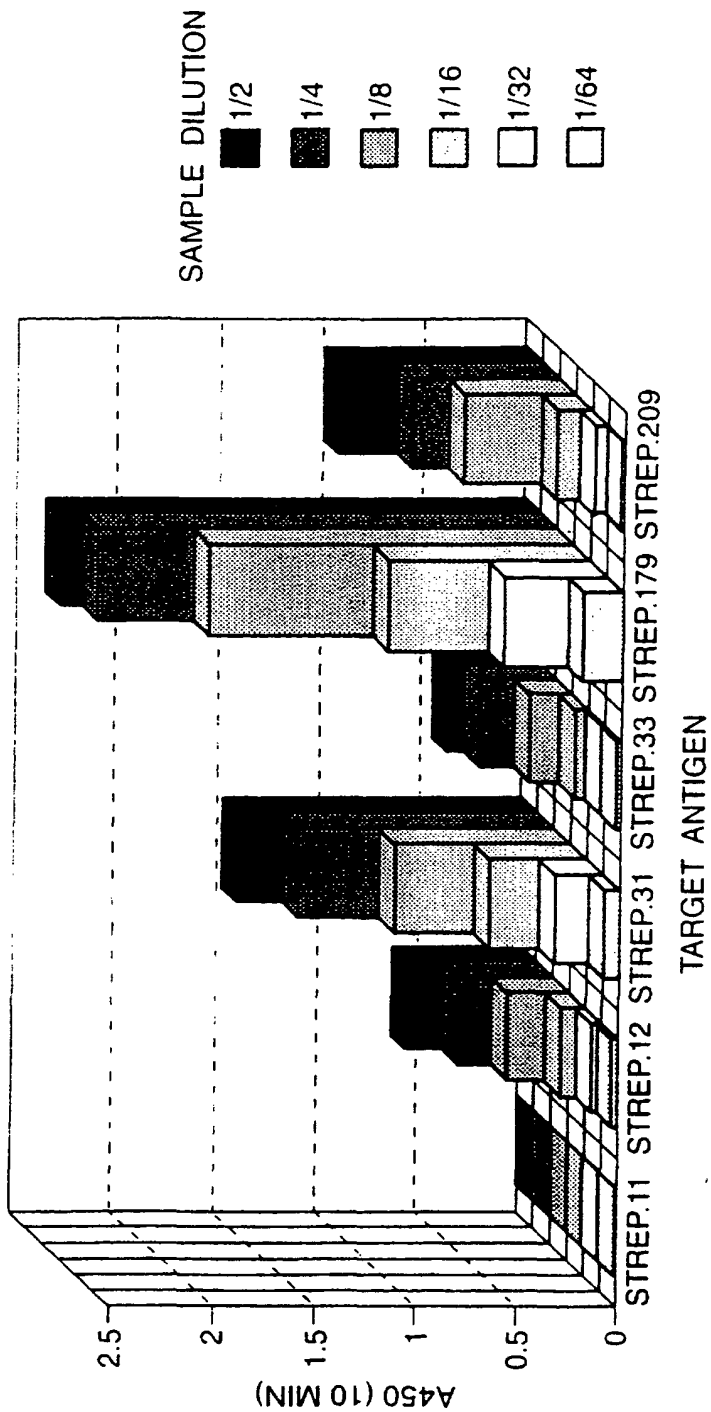
15/45

Fig.14.



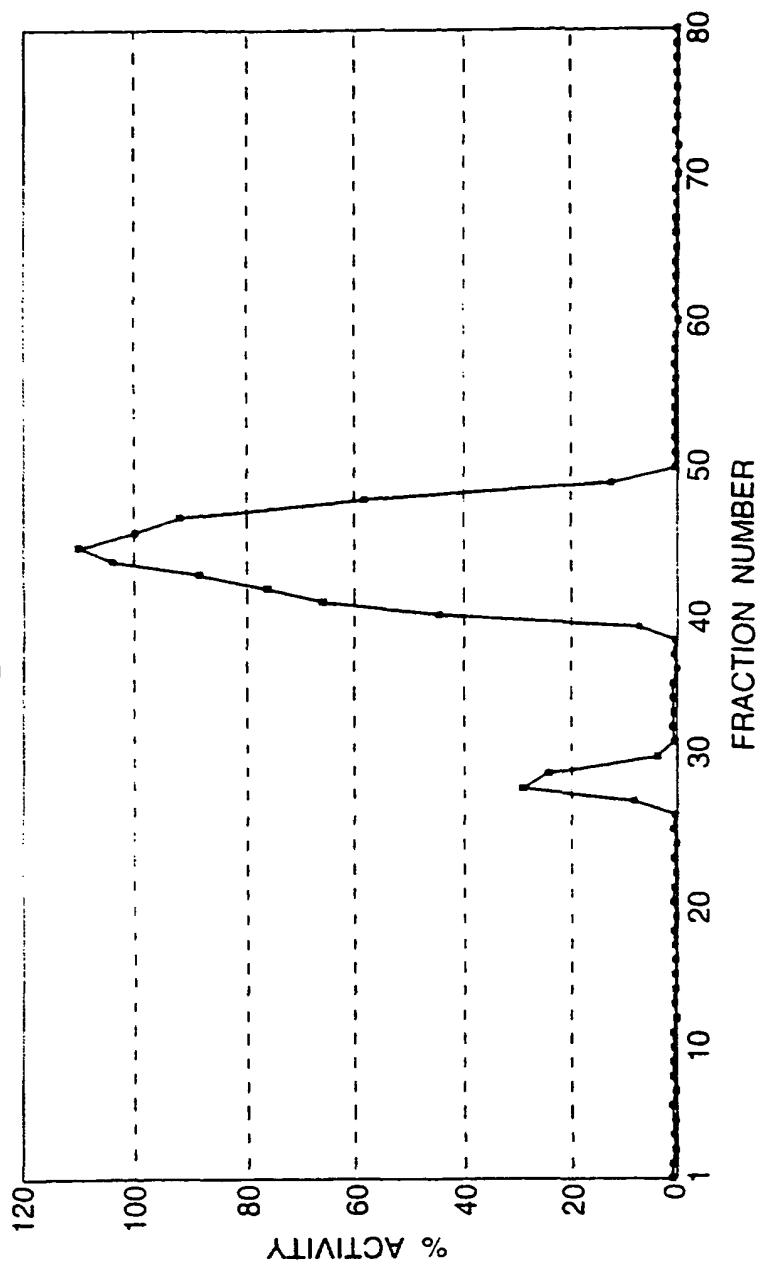
16/45

Fig.15.



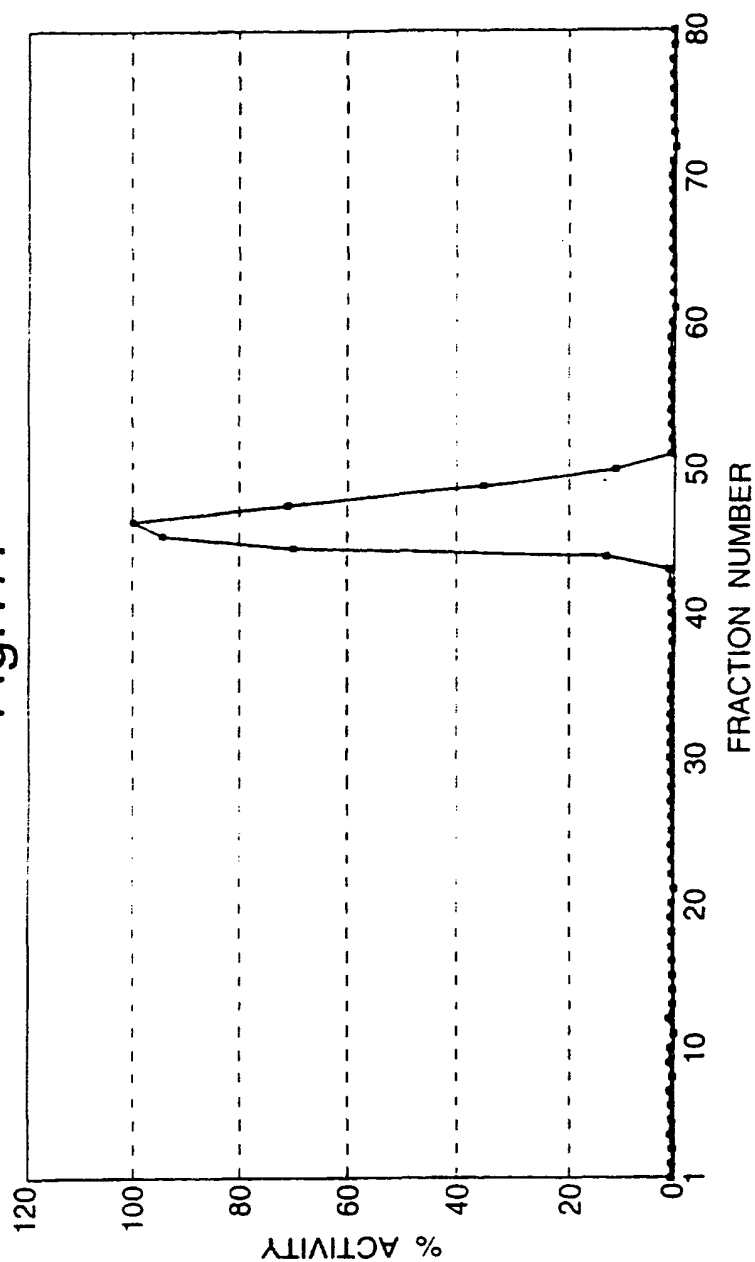
17/45

Fig.16.



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Fig.17.



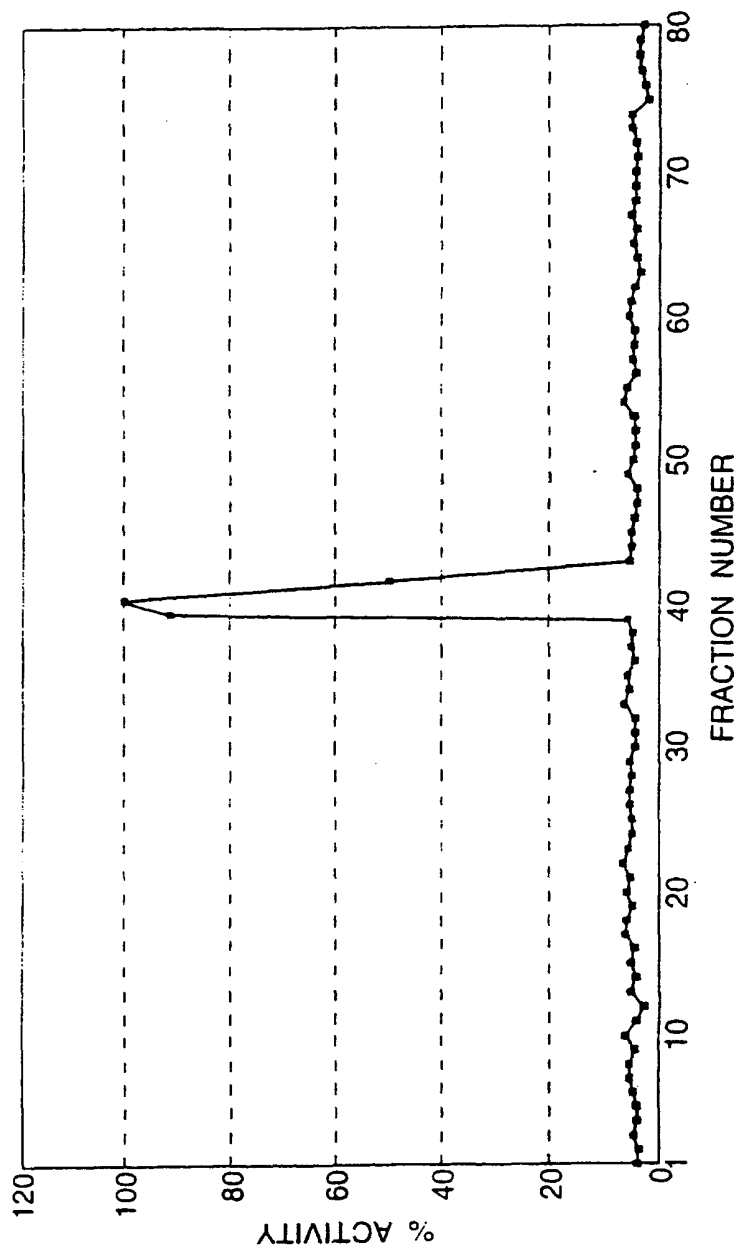
19/45

NOT TO BE TAKEN  
INTO CONSIDERATION  
FOR THE PURPOSES  
OF INTERNATIONAL PROCESSING

(See Section 310(d)(ii) of the Administrative Instructions)

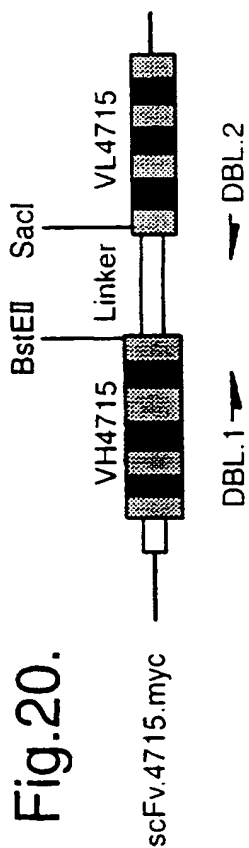
20/45

Fig.19.



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Fig.20.



DIGEST WITH  
BstEI/SacI

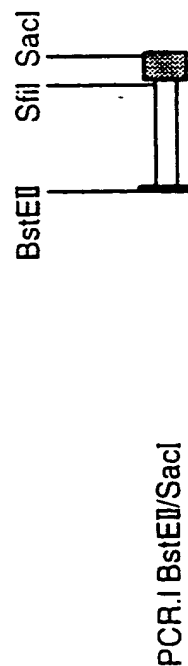
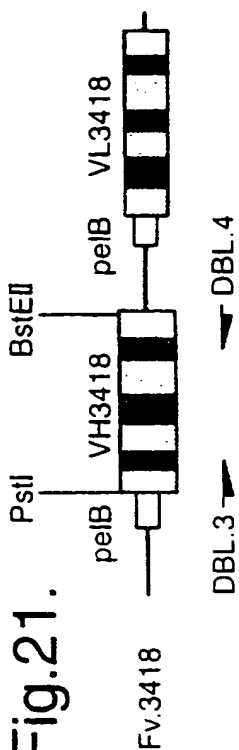


Fig.21.



DIGEST WITH  
SfiI/EcoRI





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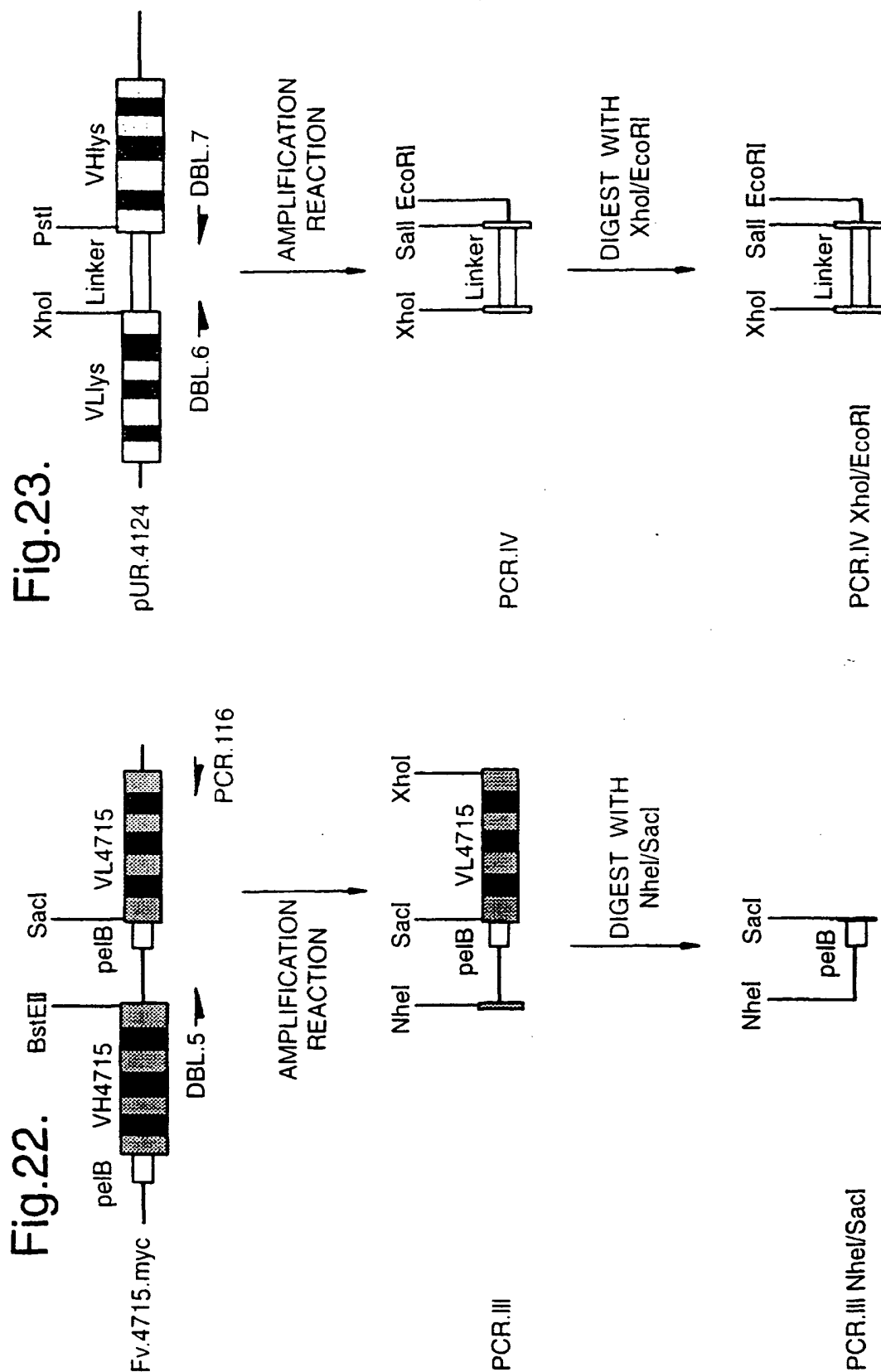


Fig.24.

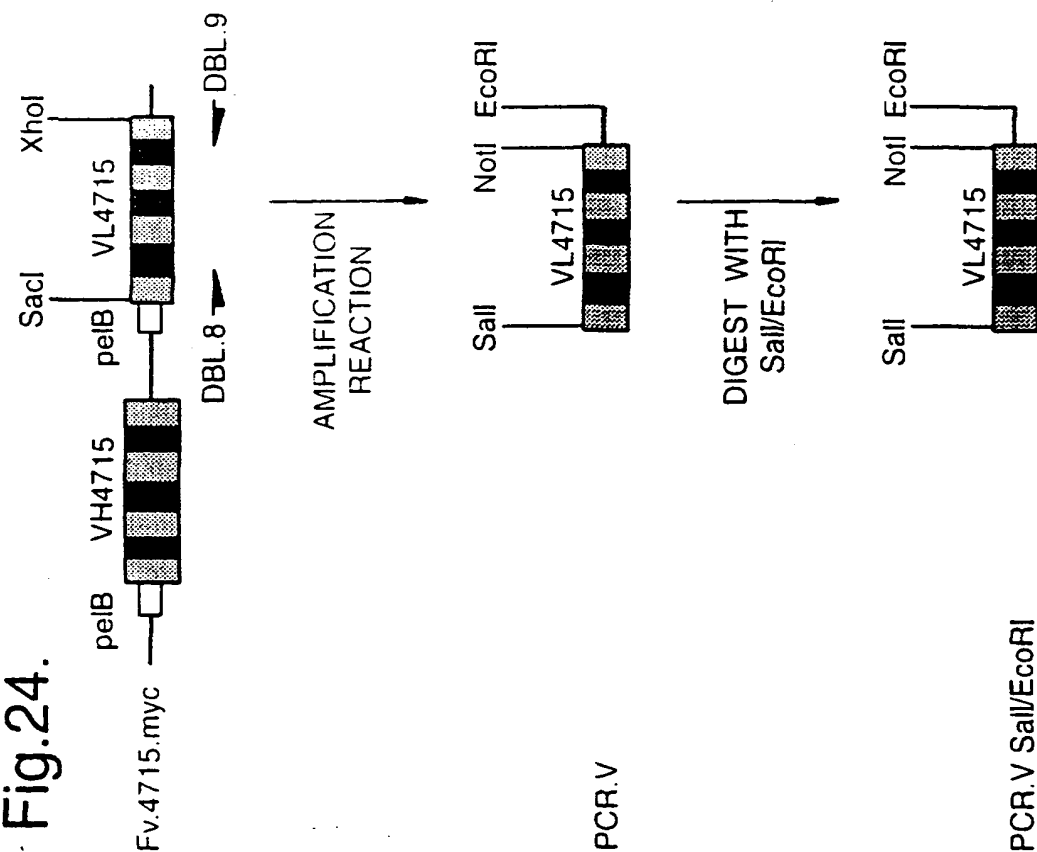
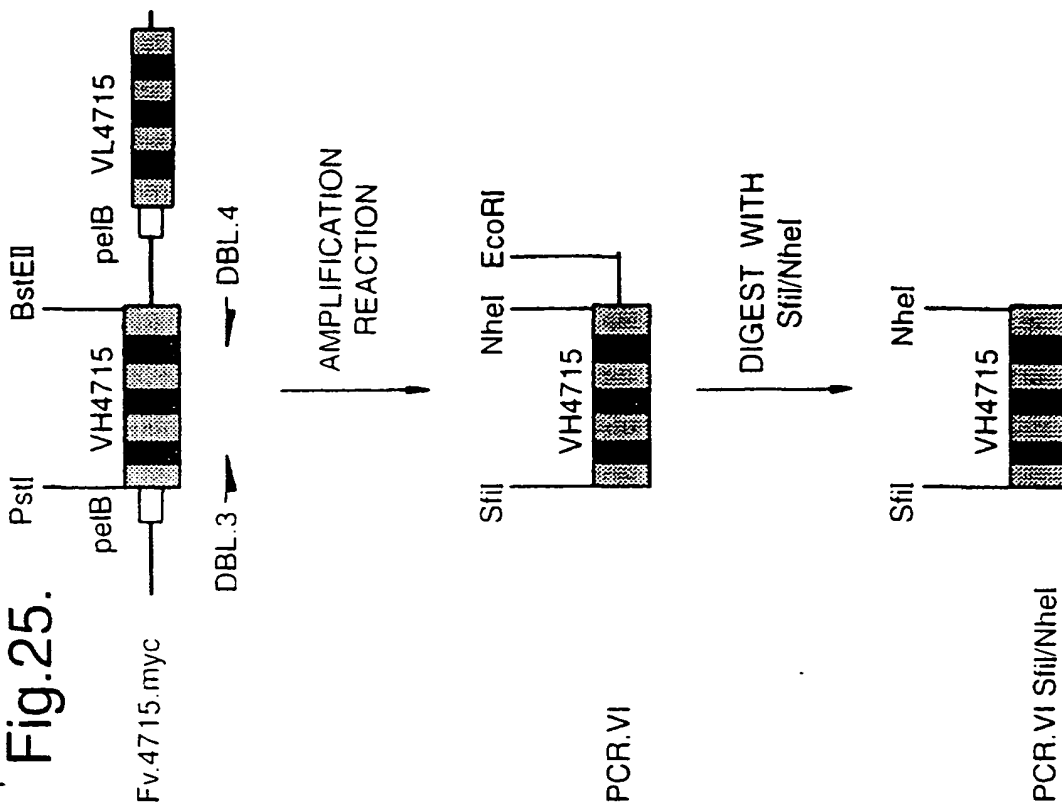


Fig.25.



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Fig.27.

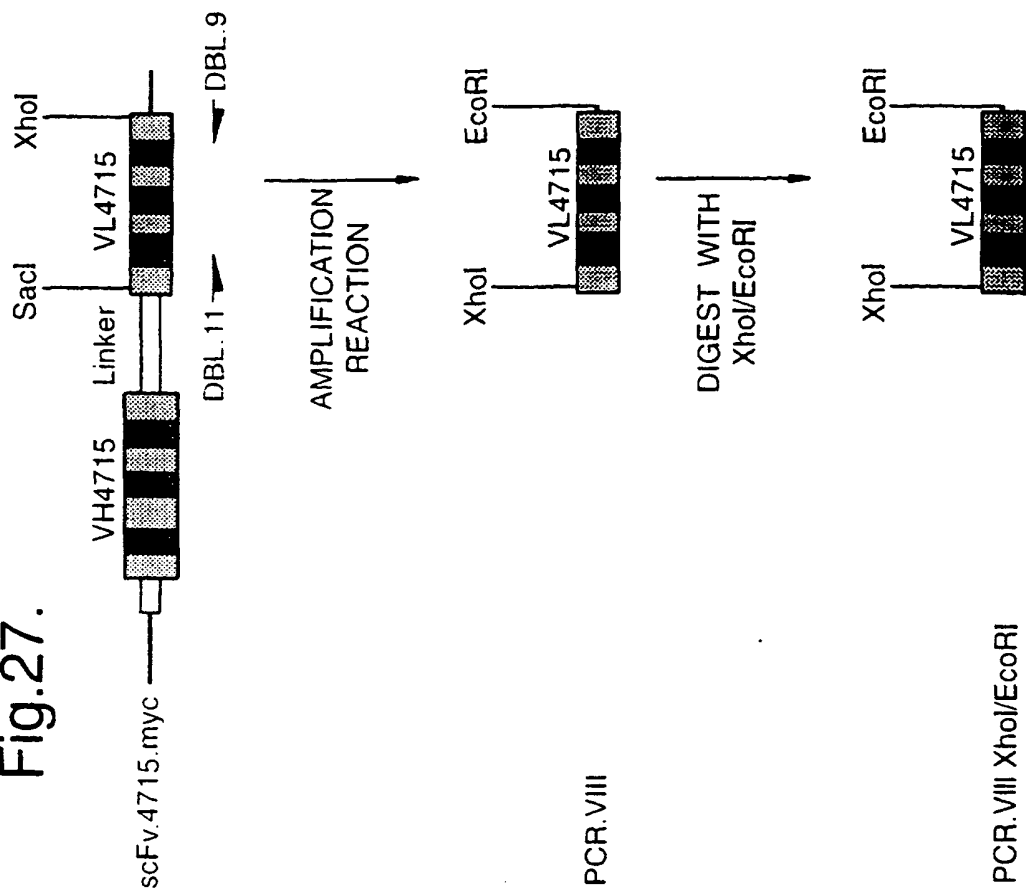
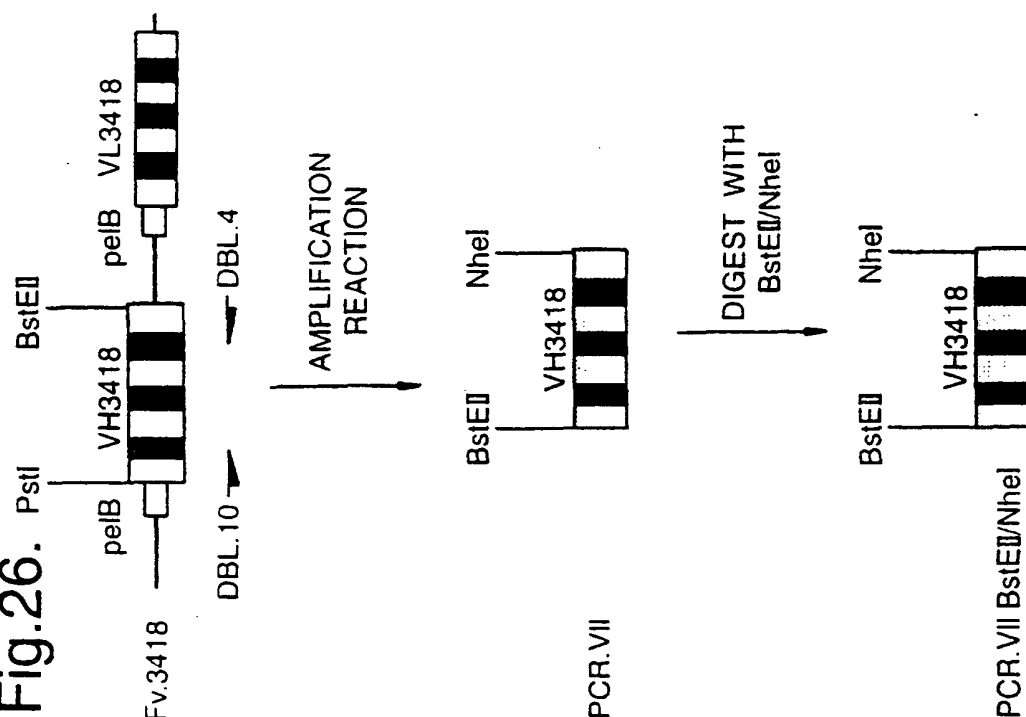
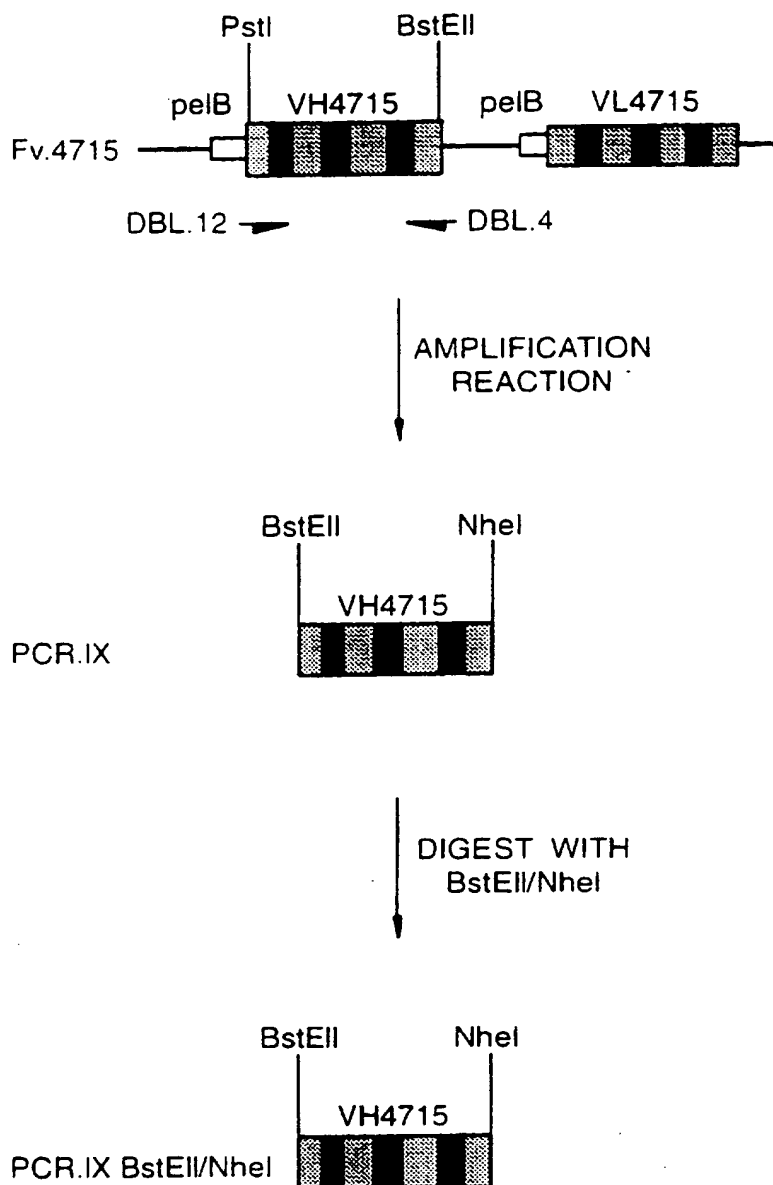


Fig.26.



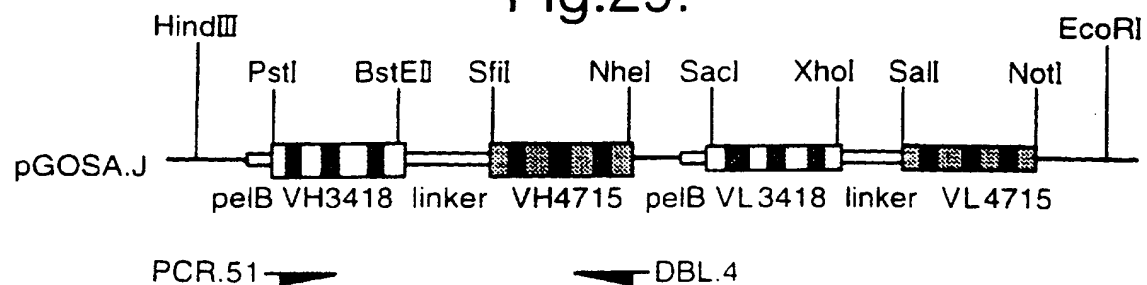
25/45

Fig.28.



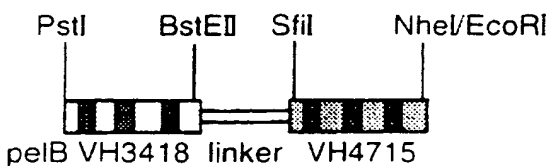
26/45

Fig.29.



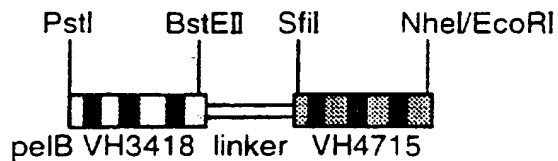
AMPLIFICATION  
REACTION

PVR.X

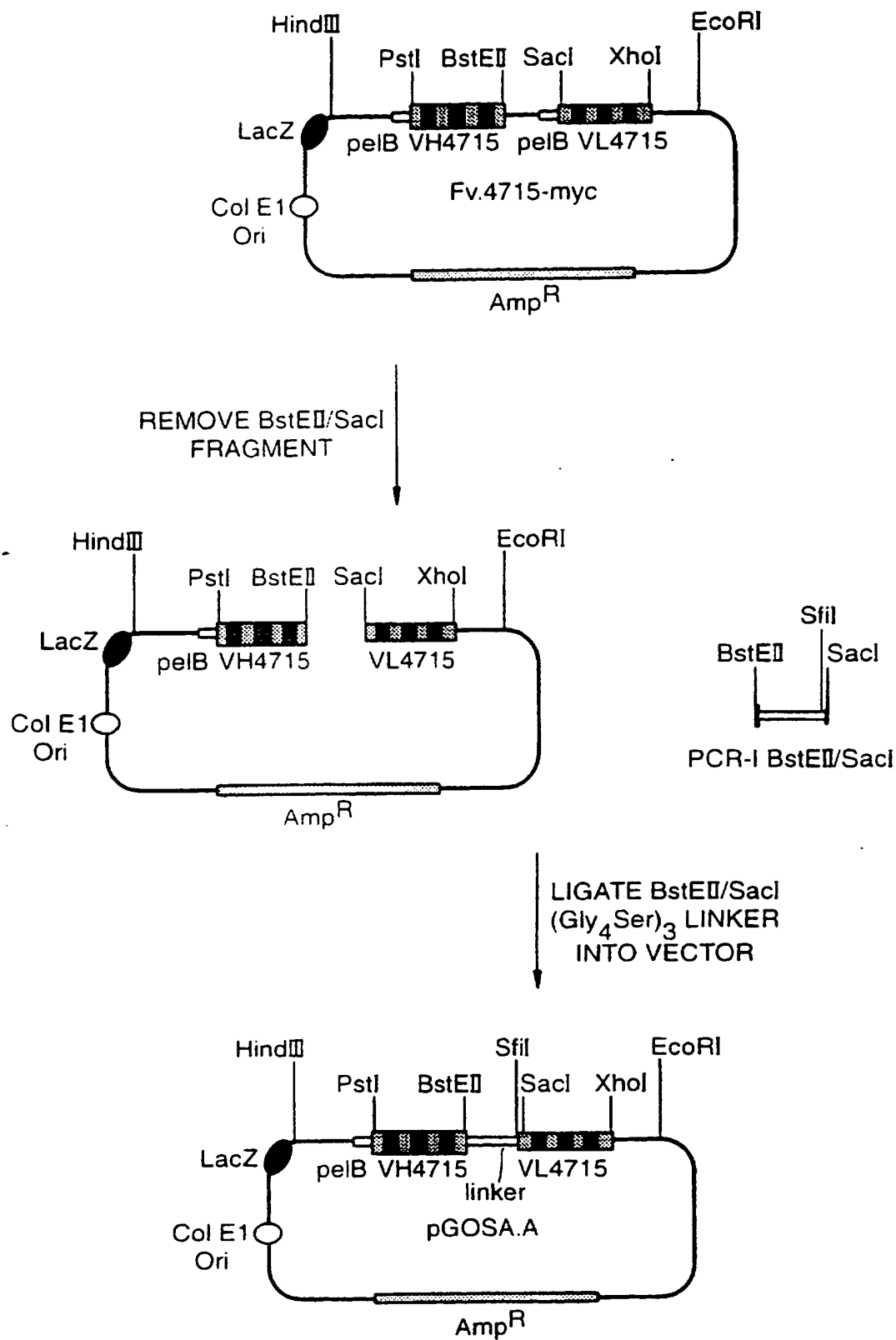


DIGEST PCR.X  
WITH PstI/EcoRI

PCR.X PstI/EcoRI

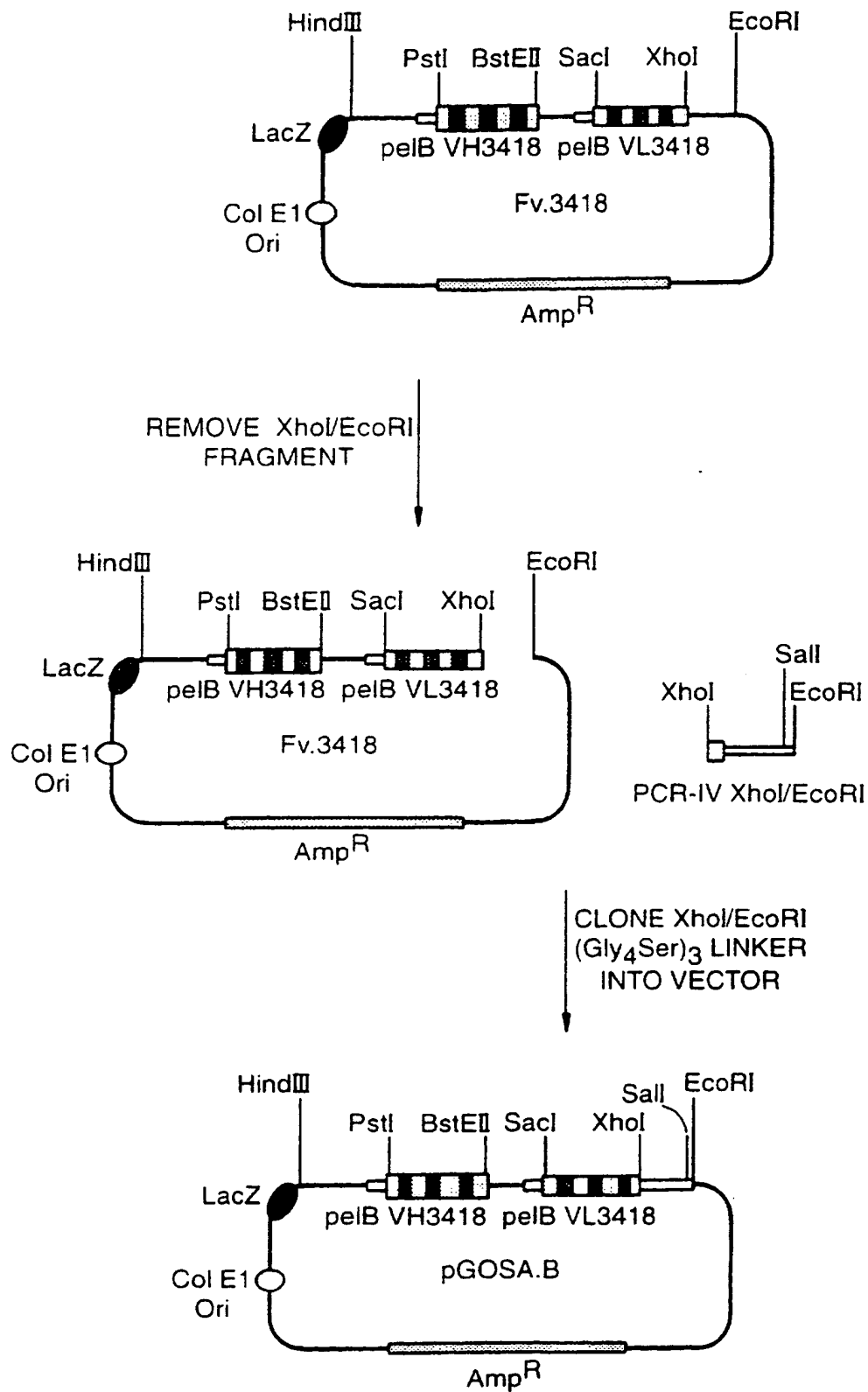


27/45  
Fig.30.



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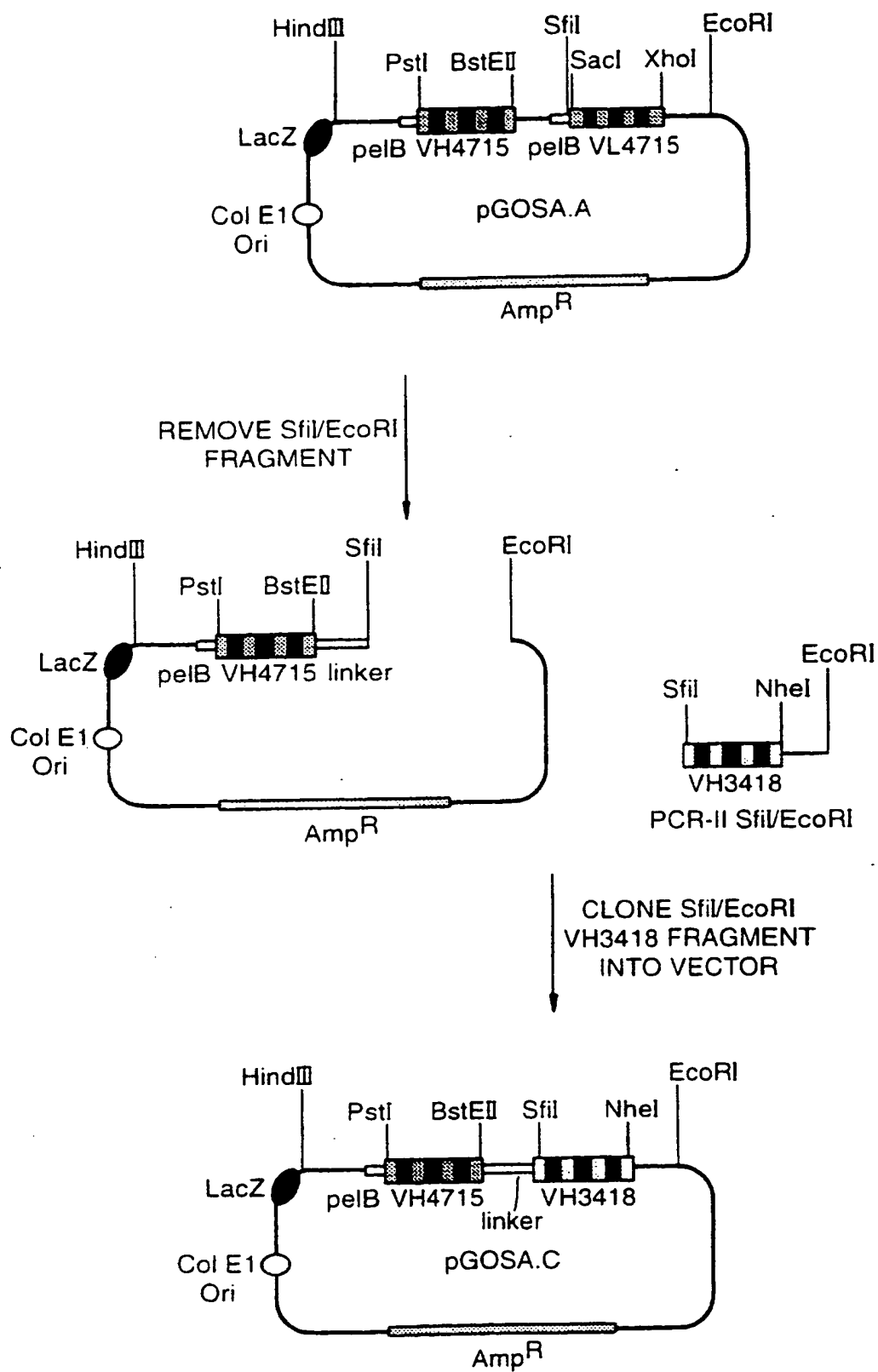
28/45  
Fig.31.



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Fig.32.

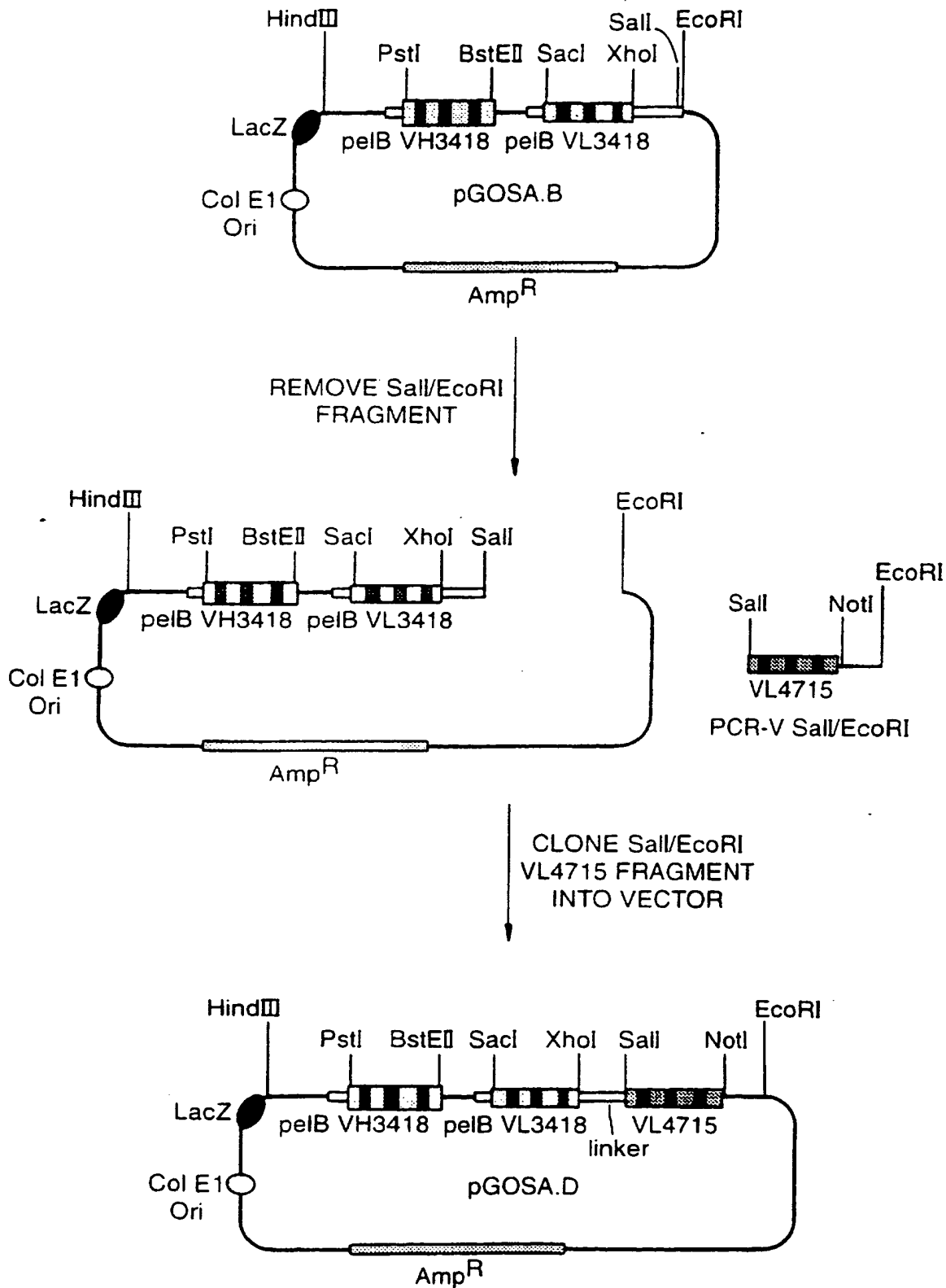


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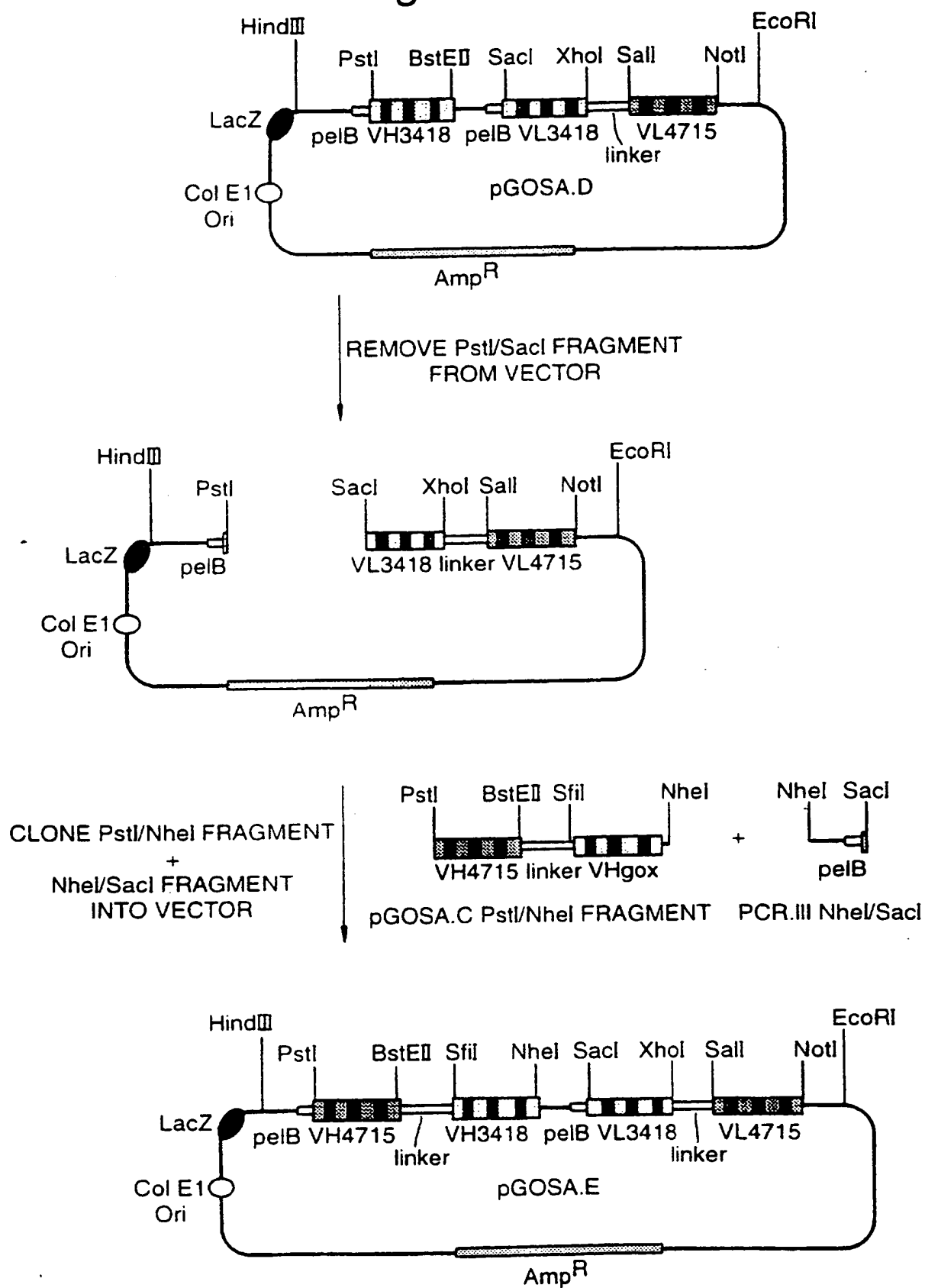


30/45

Fig.33.



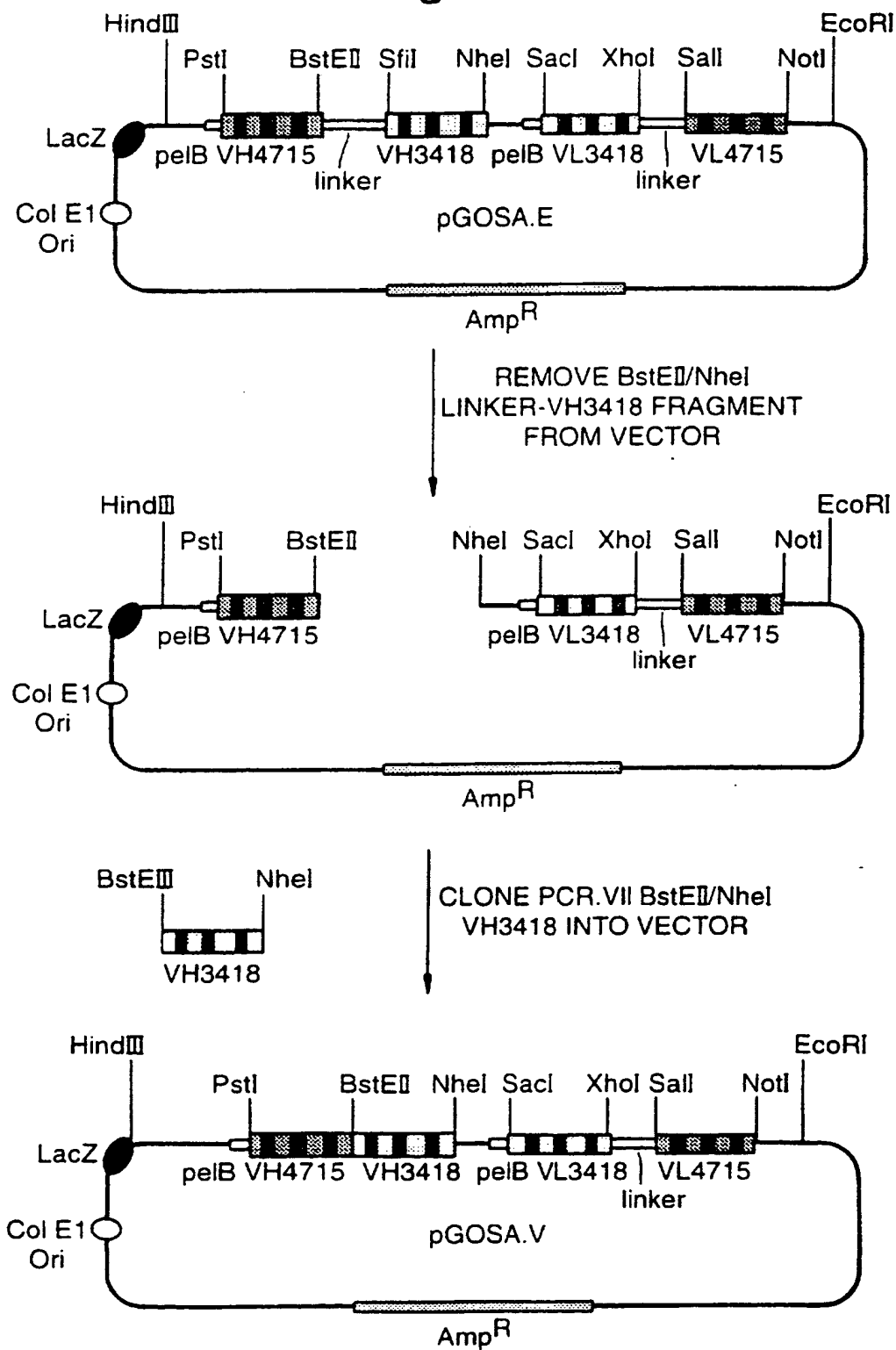
31/45  
Fig.34.



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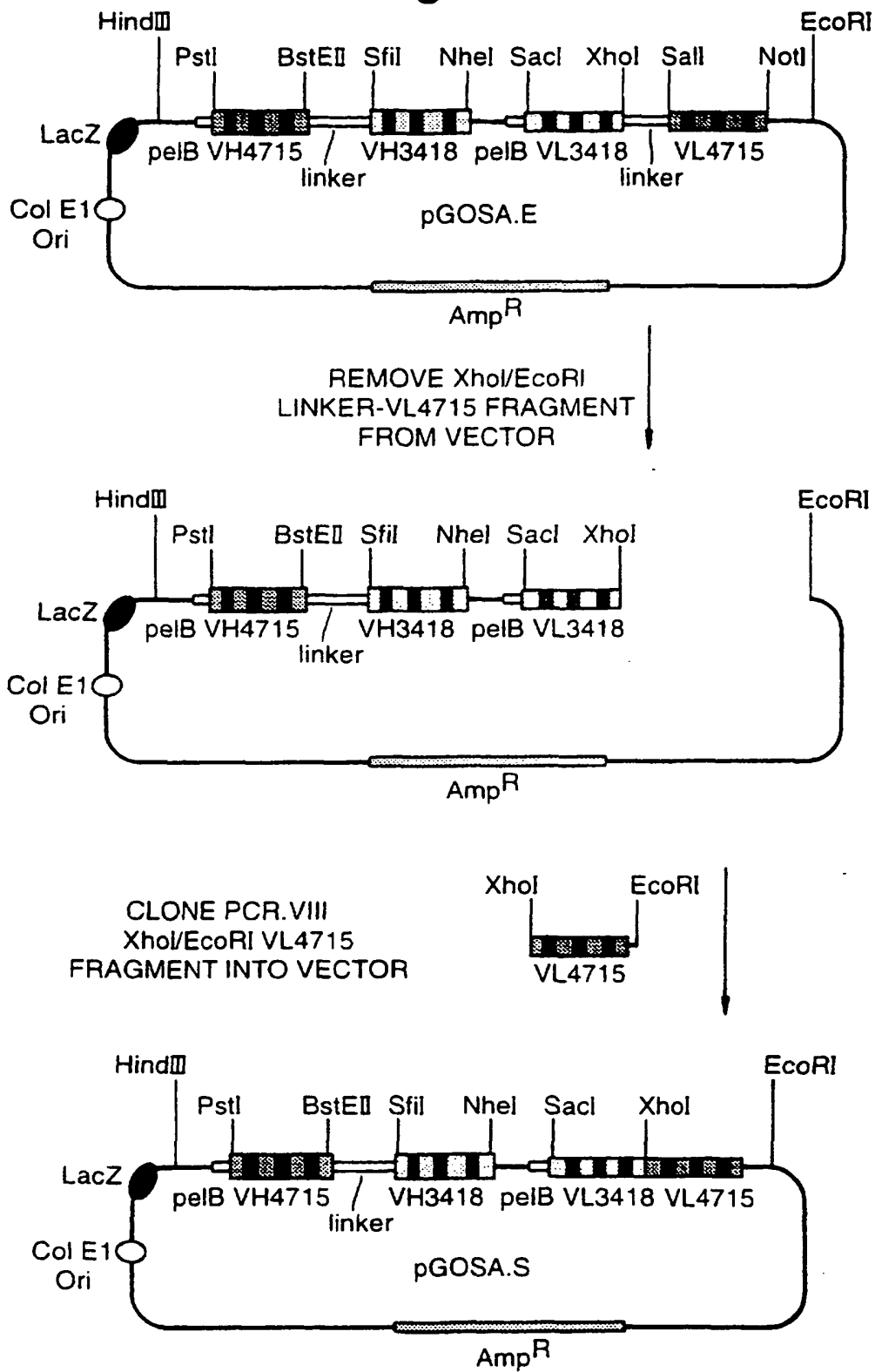
32/45

Fig.35.

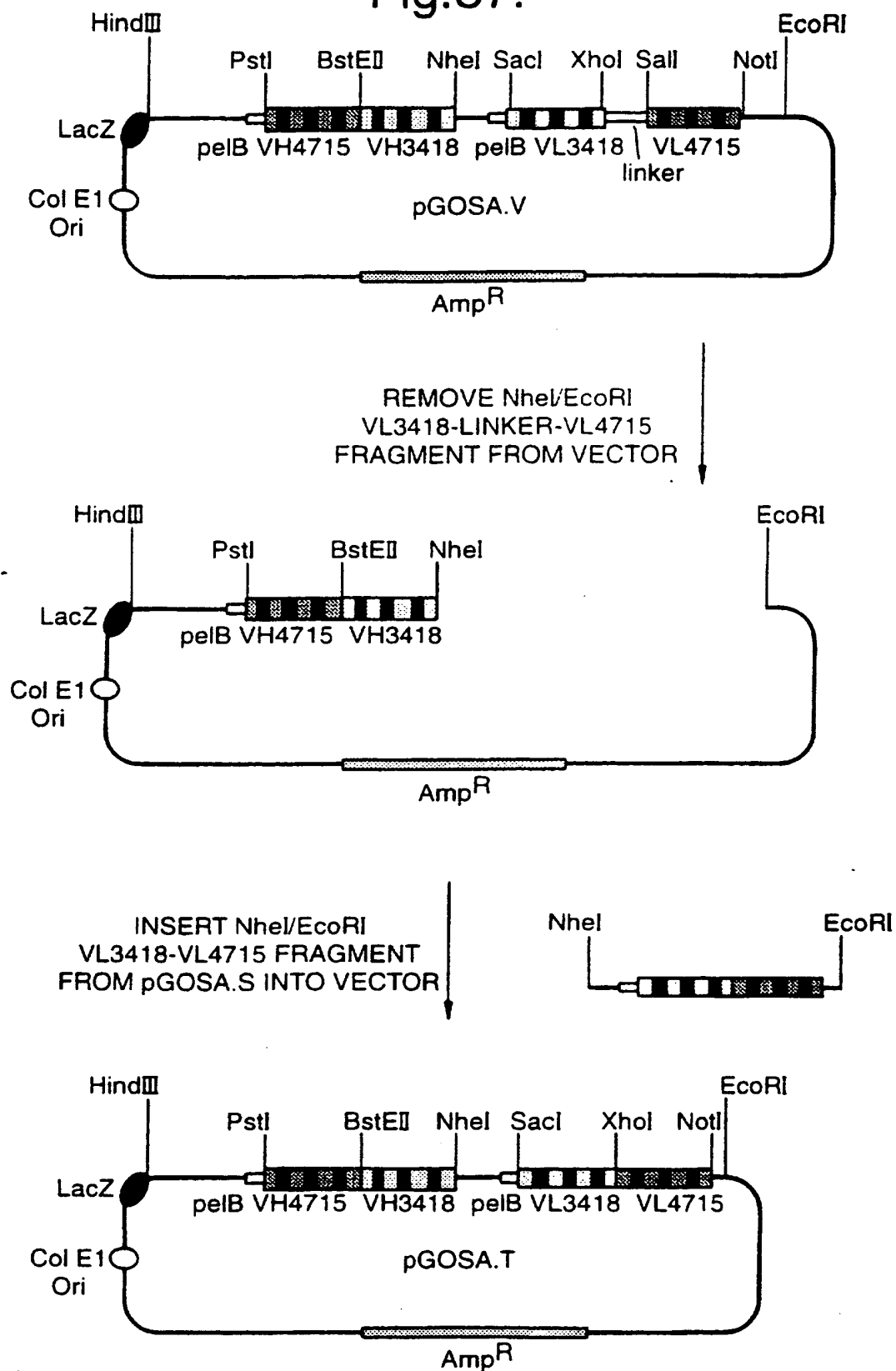


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Fig.36.



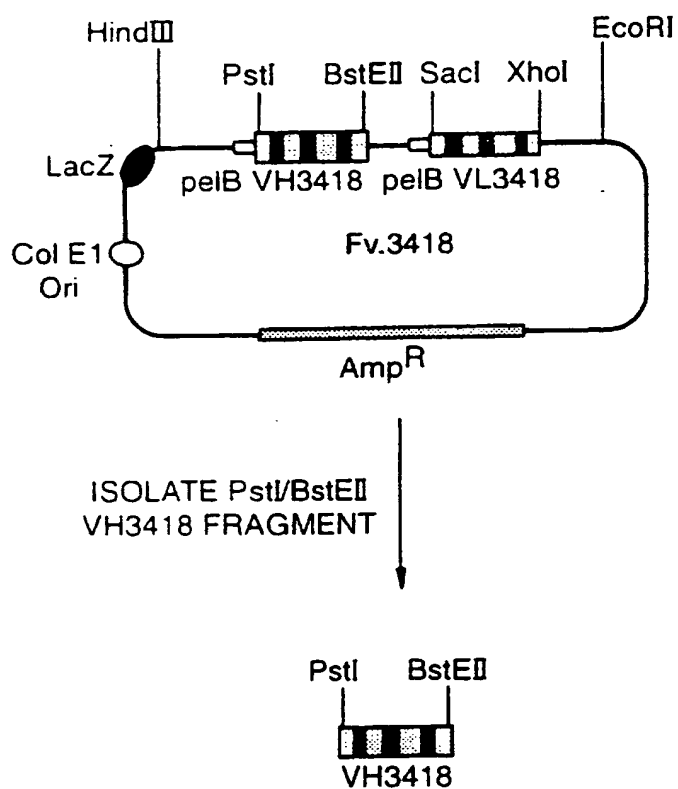
SUBSTITUTE SHEET (RULE 26)

34/45  
Fig.37.

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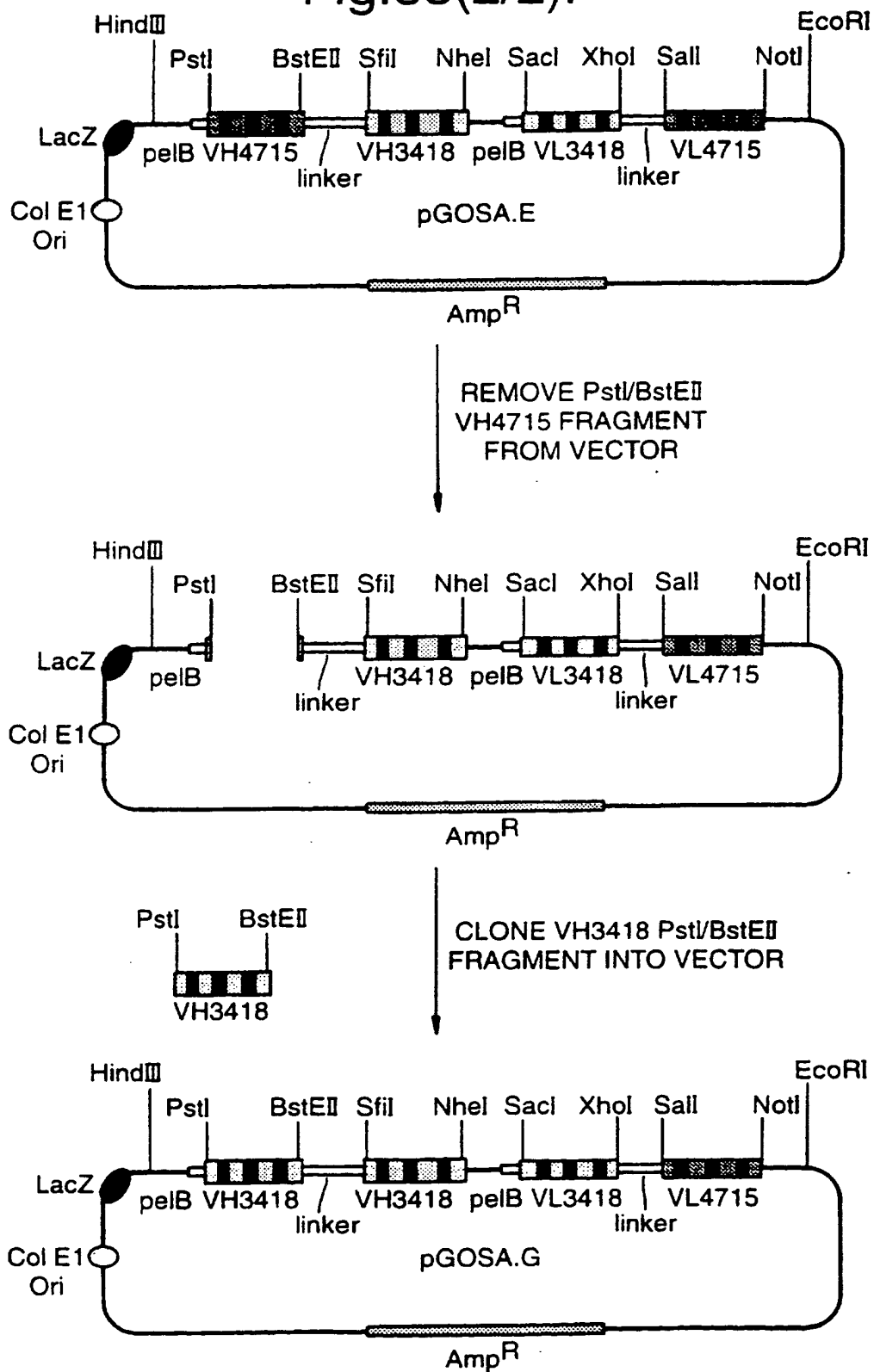
35/45

Fig.38(1/2).



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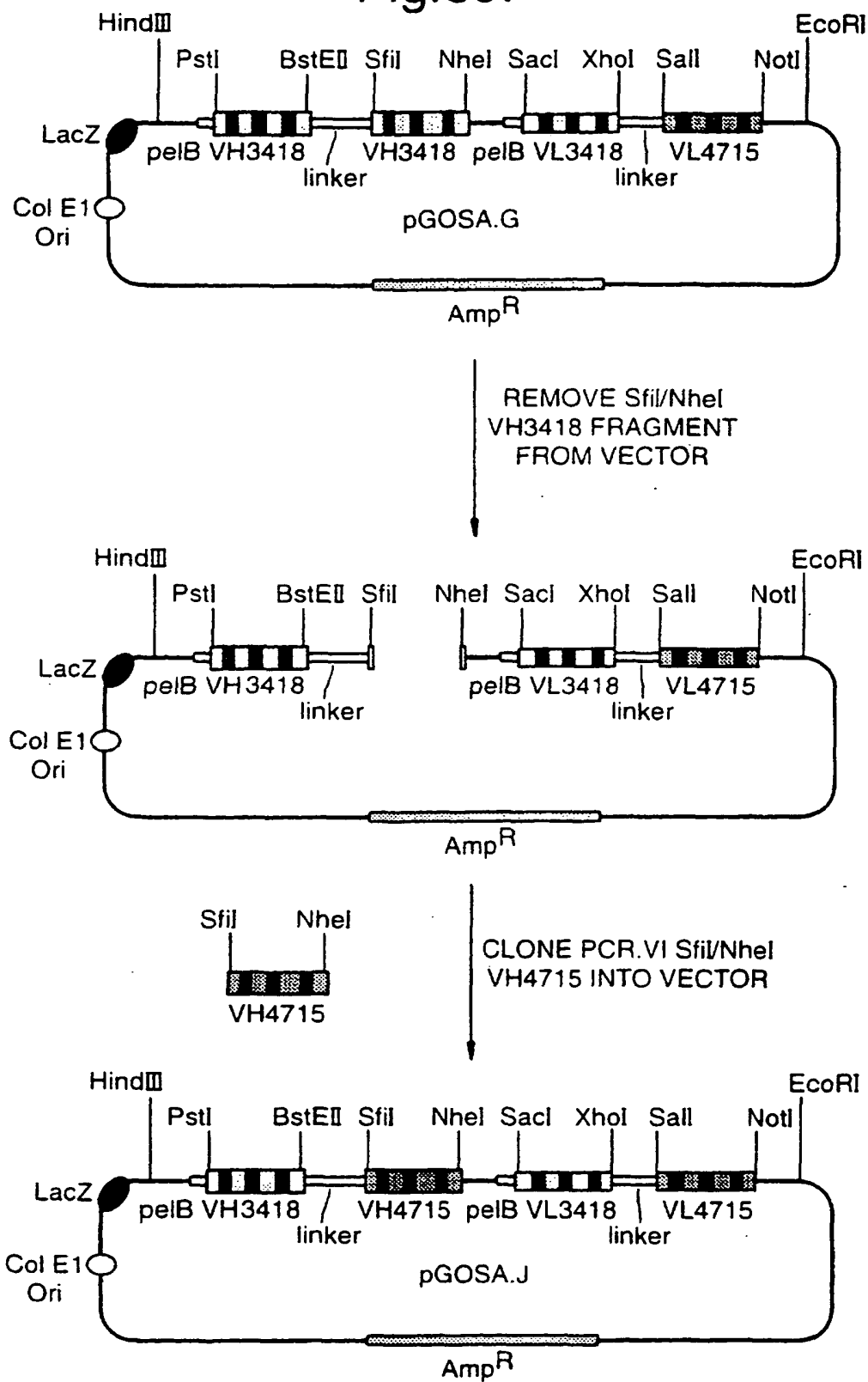
Fig.38(2/2).



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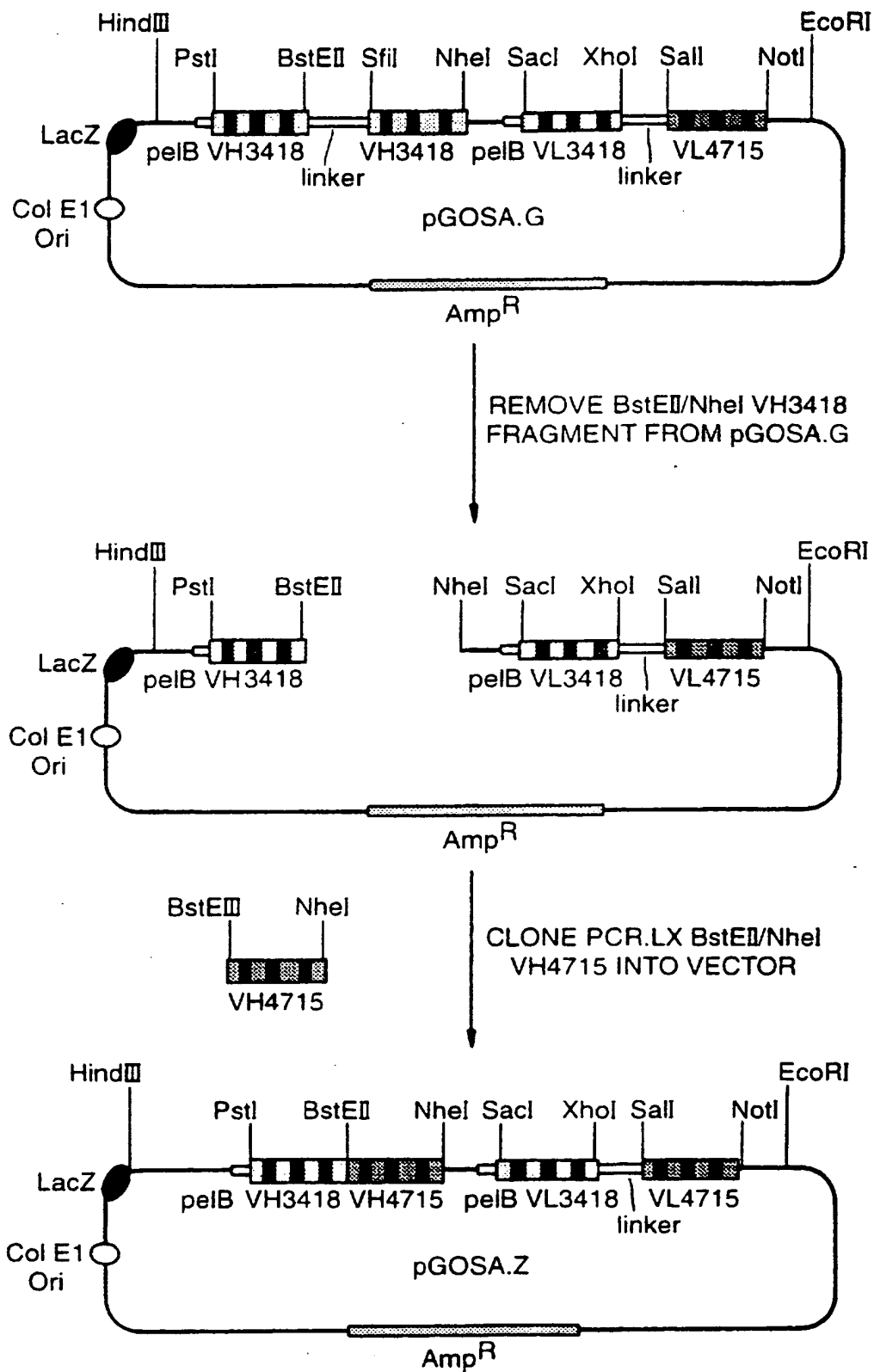
Fig.39.





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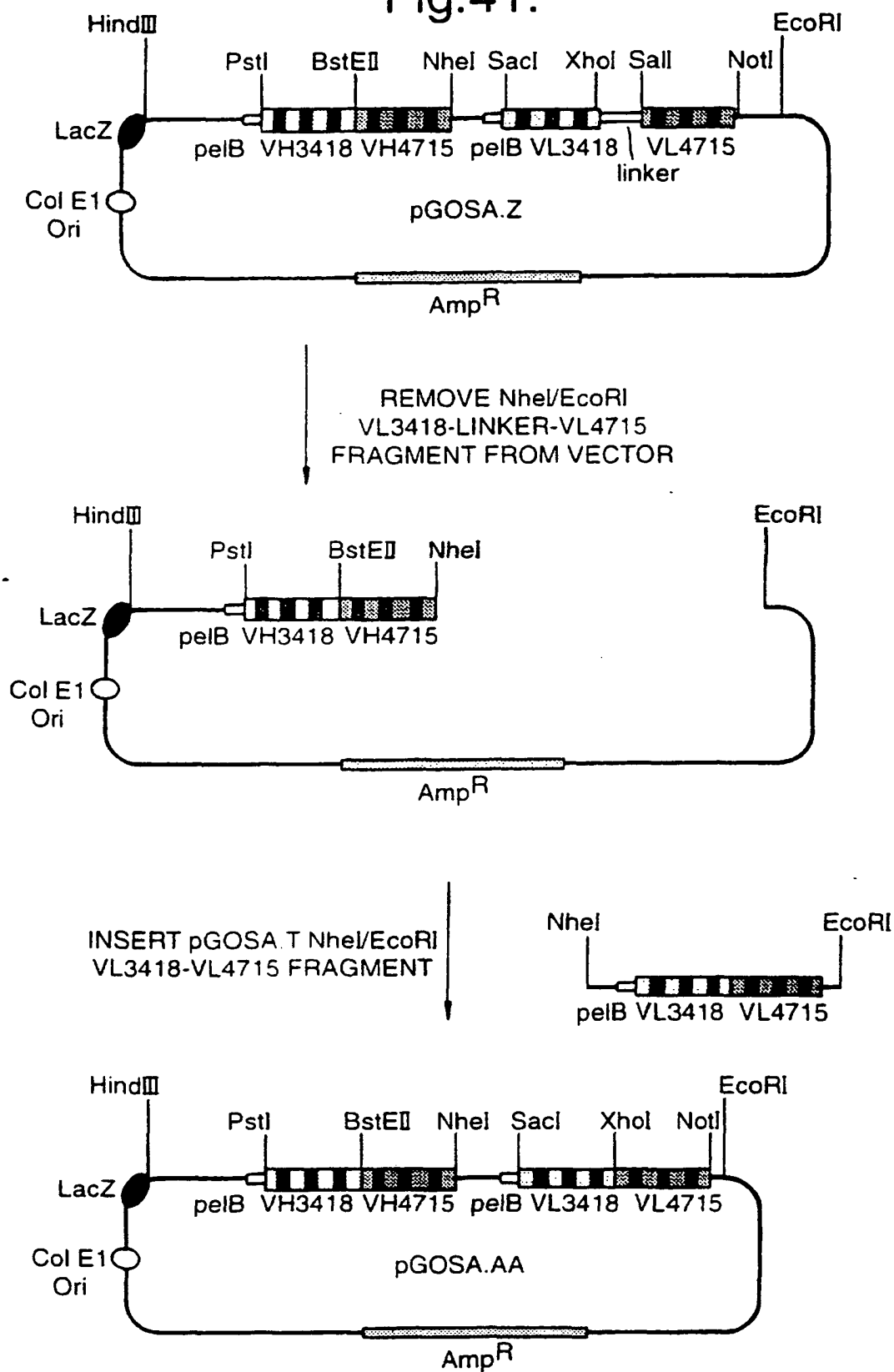
Fig.40.



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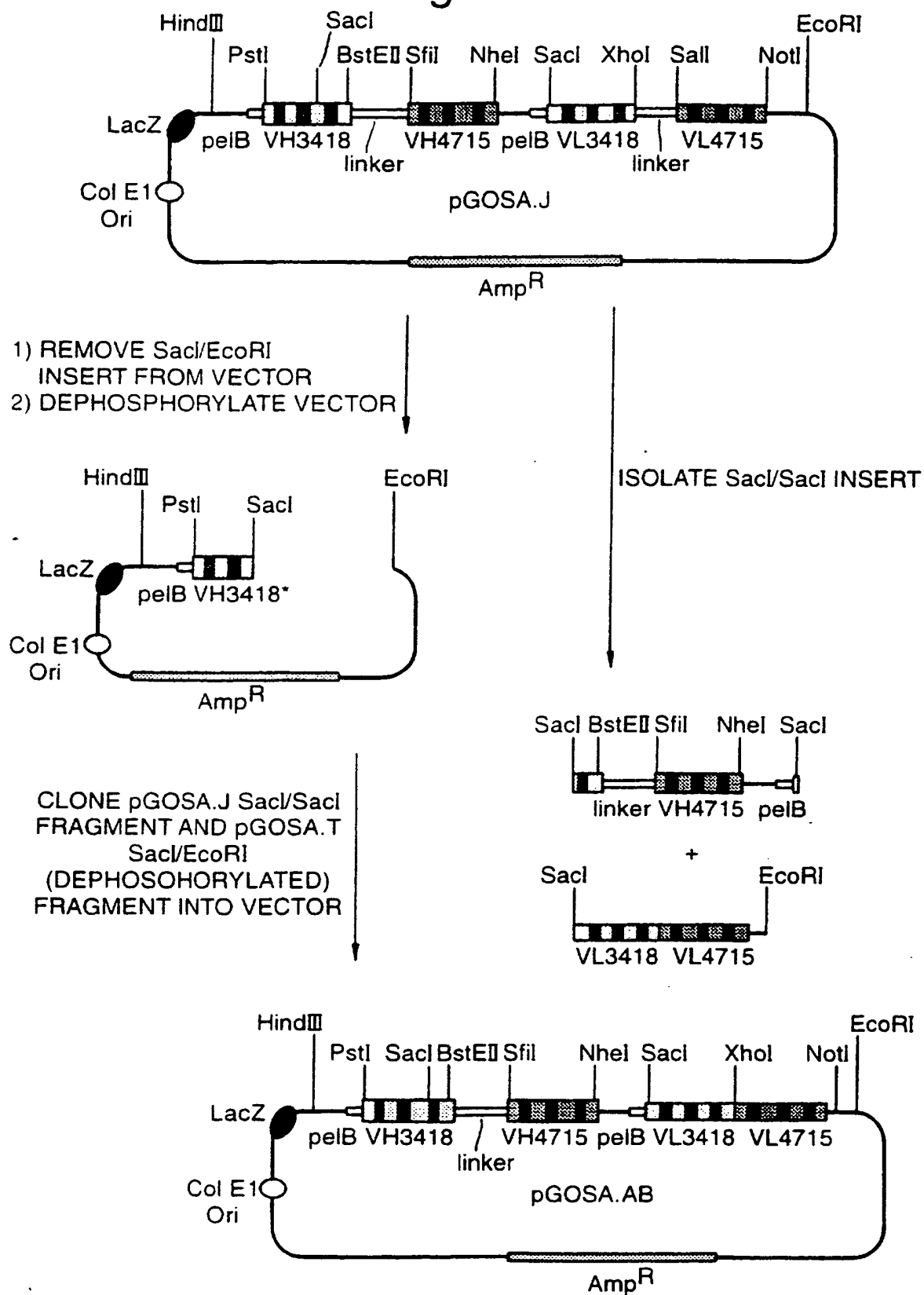
39/45

Fig.41.



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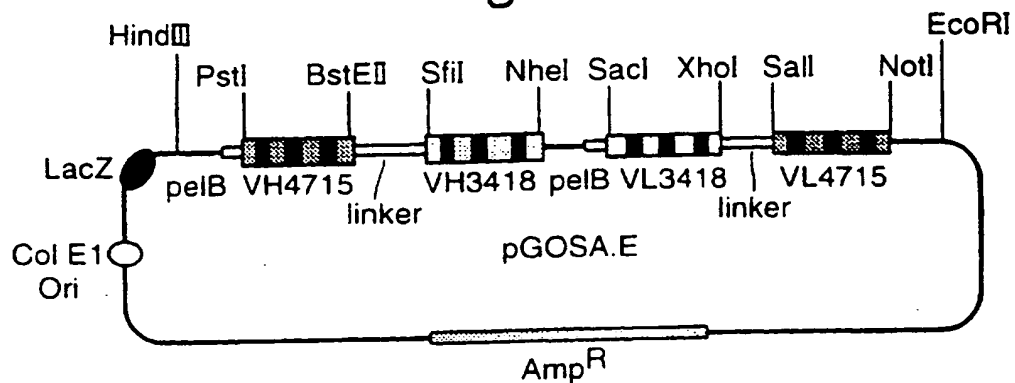
40/45  
Fig.42.



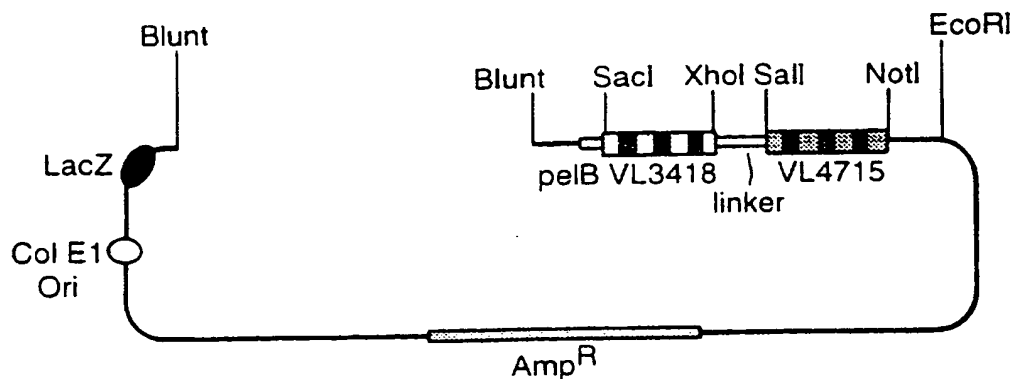
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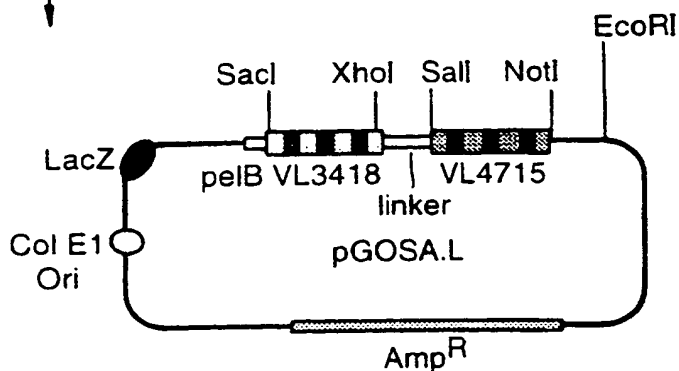
Fig.43.



- 1) REMOVE HindIII/NheI VH4715-LINKER-VH3418 FRAGMENT FROM VECTOR
- 2) FILL IN ENDS WITH KLENOW DNA POLYMERASE



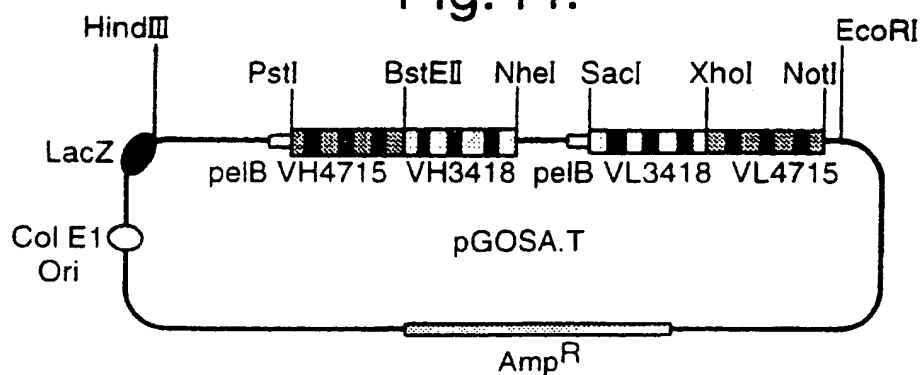
CLOSE VECTOR



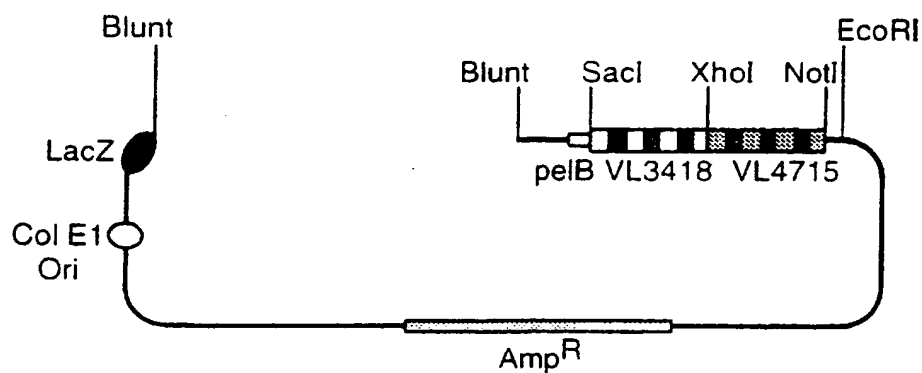
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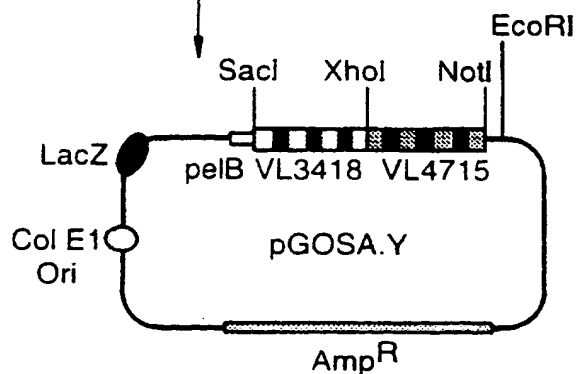
Fig.44.



- 1) REMOVE HindIII/NheI VH4715- VH3418  
FRAGMENT FROM pGOSA.T
- 2) FILL IN ENDS WITH KLENOW DNA POLYMERASE

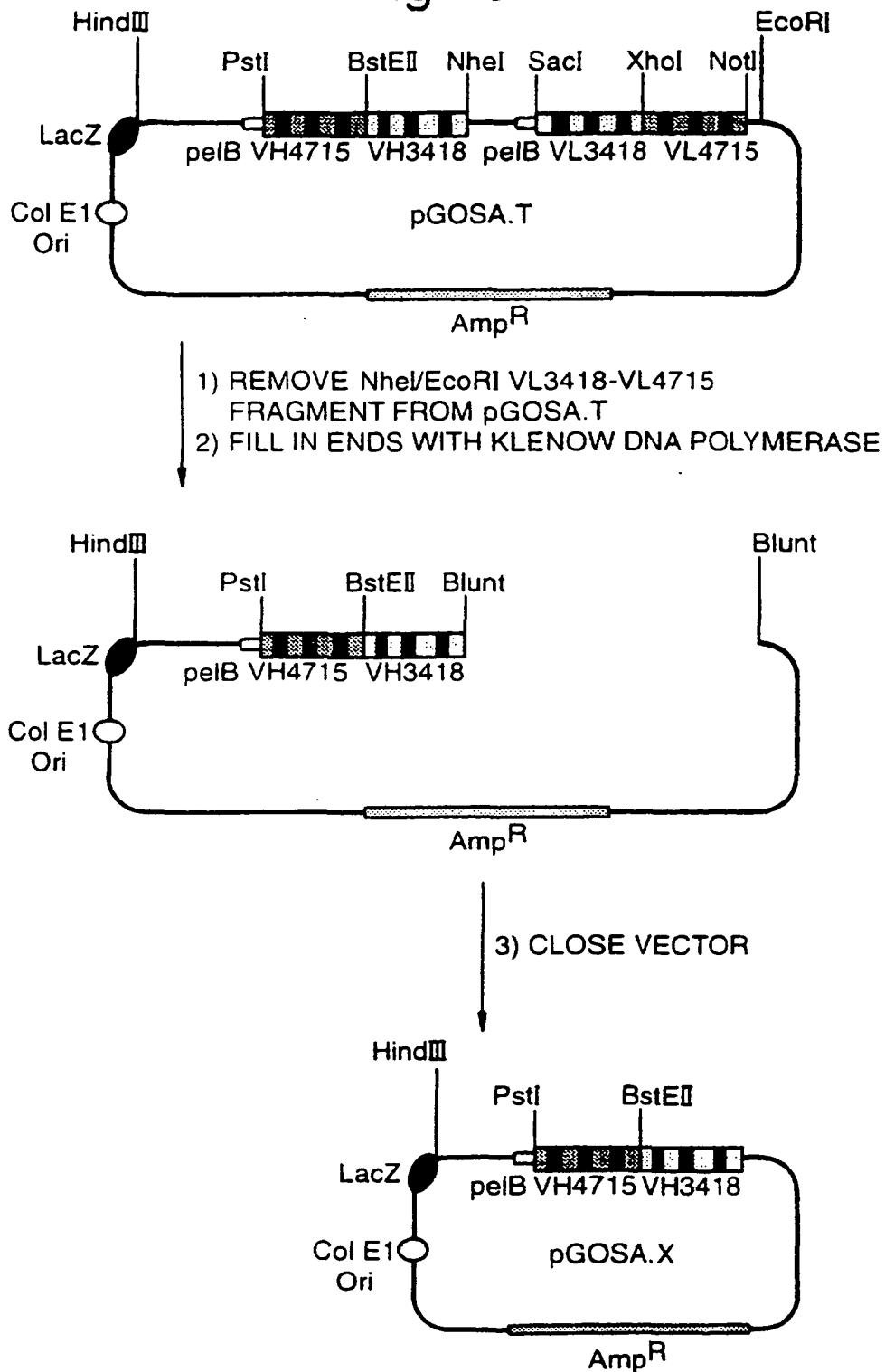


CLOSE VECTOR



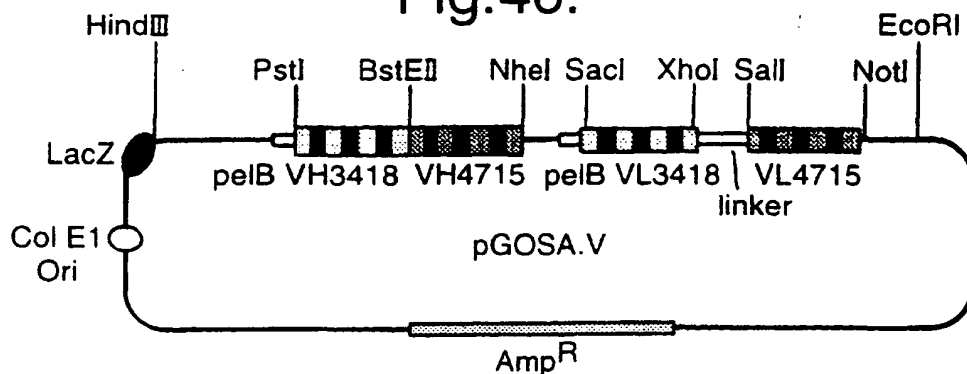
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Fig.45.

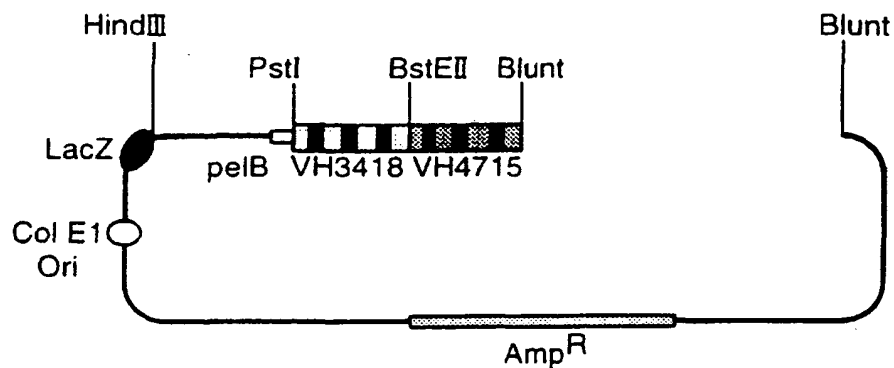


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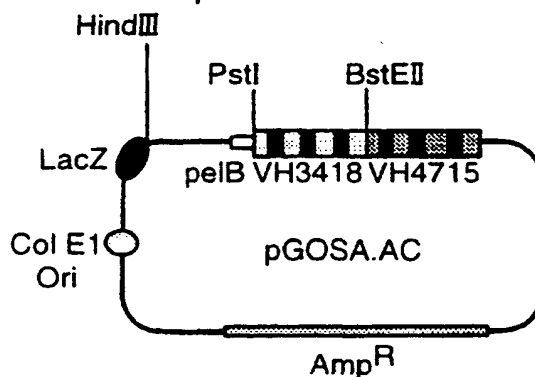
Fig.46.



- 1) REMOVE NheI/EcoRI VL3418-linker-VL4715 FRAGMENT FROM VECTOR
- 2) FILL IN ENDS WITH KLENOW DNA POLYMERASE

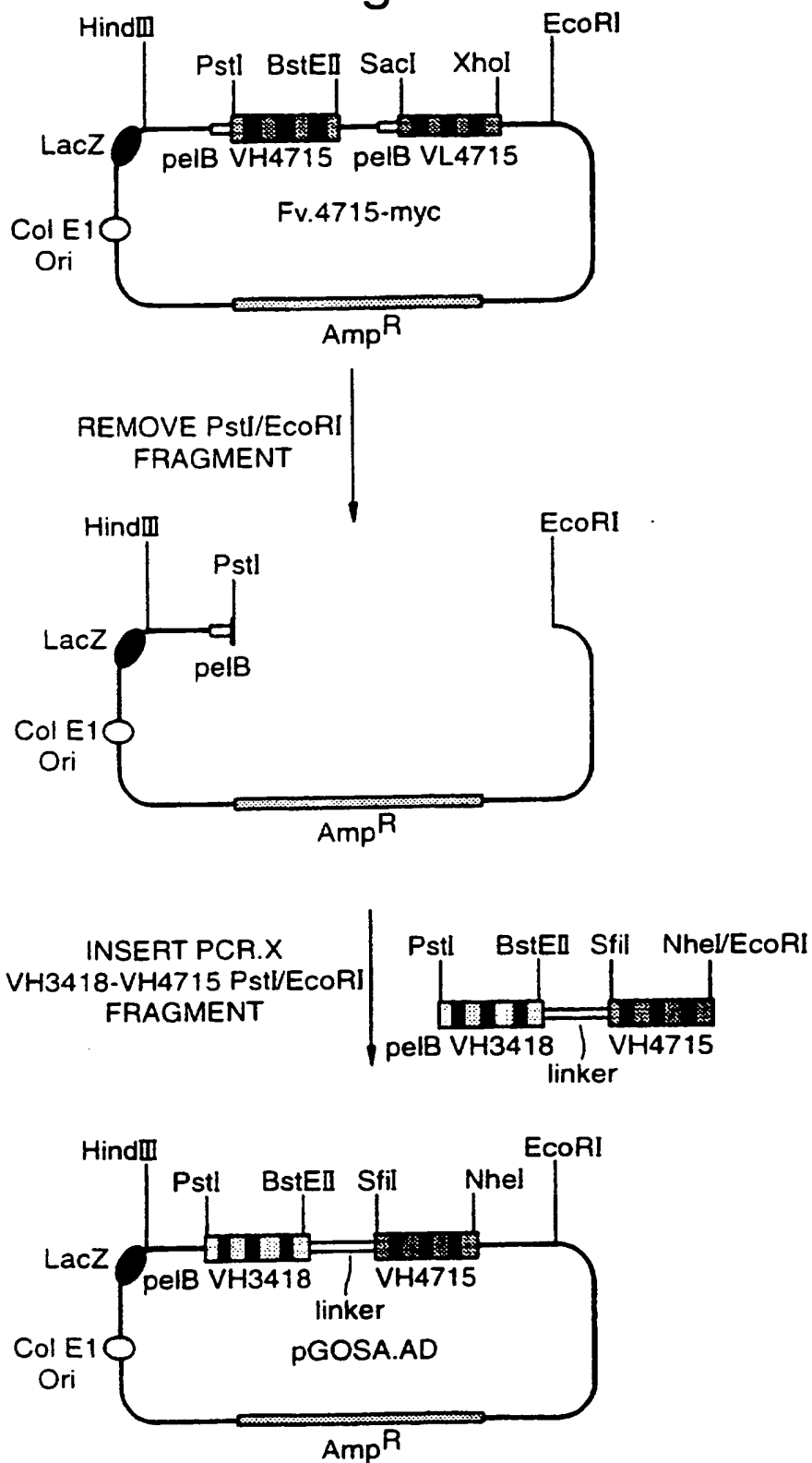


CLOSE VECTOR



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Fig.47.



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## INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/EP 96/03605

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K16/46 C07K16/00 A61K39/395 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,0	BIOCHEMICAL SOCIETY TRANSACTIONS, vol. 23, no. 4, 18 - 21 July 1995, LONDON, GB, pages 1067-1073, XP000565752 M. VERHOEYEN ET AL.: "Antibody fragments for controlled delivery of therapeutic agents." see the whole document --- -/--	1-14

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

16 December 1996

Date of mailing of the international search report

07. 01. 97

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Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No.

/EP 96/03605

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 90, no. 14, 15 July 1993, WASHINGTON, DC, USA, pages 6444-6448, XP002021302 P. HOLLIGER ET AL.: "Diabodies": Small bivalent and bispecific antibody fragments." cited in the application see the whole document</p>	1-14
A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 1, 7 January 1994, BALTIMORE, MD, USA, pages 199-206, XP002021303 W. MALLENDER ET AL.: "Construction, expression, and activity of a bivalent bispecific single-chain antibody." cited in the application see abstract see figures 1,2</p>	1-14
A	<p>WO 94 13806 A (THE DOW CHEMICAL COMPANY) 23 June 1994 cited in the application see figure 1</p>	1-14
A	<p>WO 94 13804 A (CAMBRIDGE ANTIBODY TECHNOLOGY LTD. ET AL.) 23 June 1994 cited in the application see page 31, line 10 - line 12 see figure 1</p>	1-14
A	<p>WO 93 11161 A (ENZON, INC.) 10 June 1993 cited in the application see page 22, line 1 - line 10 see claims</p>	1-14

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 96/03605

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9413806	23-06-94	AU-A- 5747794	04-07-94
		CA-A- 2117477	23-06-94
		EP-A- 0628078	14-12-94
		JP-T- 7503622	20-04-95
-----			
WO-A-9413804	23-06-94	AU-A- 5654894	04-07-94
		CA-A- 2150262	23-06-94
		EP-A- 0672142	20-09-95
		JP-T- 8504100	07-05-96
		AU-A- 7621494	10-04-95
		CA-A- 2169620	30-03-95
		EP-A- 0720624	10-07-96
WO-A- 9508577	30-03-95		
-----			
WO-A-9311161	10-06-93	AU-A- 3178993	28-06-93
		CA-A- 2122732	10-06-93
		EP-A- 0617706	05-10-94
		JP-T- 7501451	16-02-95
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